The Epidemiology and Consequences of HIV Drug Resistance

Analyses of resistance data from European cohort studies

THESIS

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Declaration

I, Anna Schultze, confirm that the work presented in this thesis is my own. Where any information is derived from other sources, I confirm that this has been appropriately referenced. The genotyping, which forms part of Chapter 4, was performed by a team at the IrsiCaixa Foundation in Badalona, Spain, under the supervision of Dr Roger Paredes. For all chapters in this thesis I was responsible for the analytical design and statistical analysis, identifying the samples for genotyping where relevant, interpreting the results and writing the chapters.

Abstract

Although the roll-out of effective combination antiretroviral therapy (cART) has brought great benefits, concerns remain regarding the development of drug resistance. This thesis uses data from the EuroSIDA cohort, the UK Collaborative HIV cohort, the UK HIV Drug Resistance Database, the ViroLAB consortium and the EU-Resist collaboration to describe trends in resistance testing, prevalence and incidence as well as the impact of drug resistance on CD4 count declines both in the presence and absence of ART.

My findings show that the proportion of people tested for resistance following virological failure in Europe is low (31.6%) and decreasing. Individuals in Eastern Europe were less likely to receive a resistance test (adjusted odds ratio=0.72, 95% confidence interval=0.55-0.94) compared with individuals in Southern Europe. However, among those who were tested, the proportion with resistance was relatively high (77.9%), indicating a potentially selective approach to resistance testing. Among individuals maintained on a failing treatment regimen with resistance to at least one drug class, I found that CD4 counts declined less steeply among individuals with NRTI resistance, the M184V, D67N or T215Y mutation in the reverse transcriptase, or either the V82A or I54V mutation in the protease. In contrast, CD4 counts declined faster among individuals with NNRTI resistance and those with the V179D or L74I reverse transcriptase mutations. A cluster of mutations, including K103N, was also associated with faster CD4 declines. No class of drug resistance or individual mutation had a large impact on the rate of CD4 decline before the start of cART.

These findings have implications for public health policy in Europe aimed at minimizing health disparities, and can be used to provide recommendations for the construction of maintenance therapies for individuals with no other treatment options. Further research into HIV drug resistance is imperative to ensure the continued success of cART.

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Abbreviations

3TC	Lamivudine
ABA	Abacavir
aHR	Adjusted Hazard Ratio
AIDS	Acquired Immunodeficiency Syndrome
AMP	Fos-Amprenavir
aOR	Adjusted Odds Ratio
aRR	Adjusted Rate Ratio
ATA	Atazanavir
AZT	Zidovudine
BHIVA	British HIV Association
CD4	Cluster of Differentiation 4
CDC	Centers for Disease Control
CI	Confidence Interval
CHIP	Copenhagen HIV Programme
CNS	Central Nervous System
CP	Copies
CRF	Case Report Form
CTU	Clinical Trials Unit
D4T	Stavudine
DAA	Direct Acting Antivirals
DAD	Data collection on Adverse events of Anti-HIV Drugs
DAG	Directed Acyclic Graph
DAR	Darunavir
DBS	Dried Blood Spots
DDI	Didanosine
DTG	Dolutegravir
EACS	European AIDS Clinical Society
EACS	European Clinical AIDS Society
EFV	Efavirenz
EMA	European Medicines Agency
ETR	Etravirine
EU	European Union
EU-TDR	European Transmitted Drug Resistance Collaboration
FDA	Food and Drug Administration
FDR	False Discovery Rate
FTC	Emtricitabine
FU	Follow-Up
FUS	Enfuviritide
GALT	Gut Associated Lymphoid Tissue
GEE	Generalised Estimating Equations
GRT	Genotypic Resistance Test
GSS	Genotypic Sensitivity Score
HAART	Highly Active Anti-Retroviral Therapy
HDAC	Histone Deacetylase
HIV	Human Immunodeficiency Virus

HR	Hazard Ratio
IAS	International AIDS Society
IC	Inhibitory Coefficient
IND	Indinavir
INI	Integrase Inhibitor
INSTI	Integrase Strand Transfer Inhibitor
ITT	Intention To Treat
KS	Karposis Sarcoma
KM	Kaplan Meier
LAV	Lymphadenopathy Associated Virus
LOD	Limit of Detection
LOP	Lopinavir
LTFU	Loss To Follow-Up
LTNP	Long-term non-progressors
LTR	Long Terminal Repeats
MeSH	Medical Subject Heading
MI	Myocardial Infarction
MI	Multiple Imputation
mL	millilitre
mm	millimeter
MRC	Medical Research Council
MSM	Men who have Sex with Men
MTCT	Mother To Child Transmission
MVC	Maraviroc
NEV	Nevarapine
NGS	Next Generation Sequencing
NIH	National Institutes of Health
NNRTI	Non-nucleoside Reverse Transcriptase Inhibitor
NRTI	Nucleoside Reverse Transcriptase Inhibitor
OBT	Optimized Background Therapy
OR	Odds Ratio
OST	Opioid Substitution Therapy
PCP	Pneumocystis Carnii Pneumonia
PEP	Post-Exposure Prophylaxis
PI	Protease Inhibitor
PLWH	People Living With HIV
PP	Per Protocol
PR	Protease (gene)
PrEP	Pre-Exposure Prophylaxis
PWID	Person who injects drugs
PYFU	Person Years of Follow-Up
RAL	Raltegravir
RC	Replicative Capacity
RCT	Randomized Controlled Trial
RPV	Riplivirine
RR	Rate Ratio
RT	Reverse Transcriptase (gene)
RVL	Raised Viral Load (group)
SAQ	Saquinavir

SC	Steering Committee
SVL	Suppressed Viral Load (group)
TAF	Tenofovir Alafenamide
TAM	Thymidine Analogue Resistance Mutations
TC	Teleconference
TCR	Triple Class Resistance
TDF	Tenofovir
TIP	Tipranavir
UK	United Kingdom
VF	Virological Failure
VL	Viral Load
WHO	World Health Organisation
WT	Wild Type

"Did the solution become the problem? I think the answer is no. The problem is the way we use the solution."

Paul Farmer from Partners in Health, commenting on drug resistance

Interviewed for the Academy of Achievement (2009)

Chapter 1 . Background

1.1. Introduction and Thesis Objectives

The face of the global HIV epidemic has changed considerably since the first cases of acquired immunodeficiency syndrome (AIDS) were described. Following the development of combination antiretroviral therapy (cART), people living with HIV now have a life-expectancy similar to that of the general population. It is without doubt that the roll-out of cART has brought invaluable benefits, but it has also given rise to a number of new challenges. As people develop resistance to drugs in their treatment regimen, they may have to switch to costlier second or third line regimens. Transmitted drug resistance (TDR) can limit the effectiveness of first-line treatment options, and requires continuous surveillance and monitoring to inform drug resistance testing strategies. Despite its importance, many features of HIV drug resistance are still not well understood.

The aims of this PhD thesis were to describe the epidemiology of HIV drug resistance in Europe and to estimate the effect of drug resistance on markers of clinical progression. This was done through five studies, which form the results chapters of my thesis. Although a large number of potential analyses could have been done to address my aims, I chose to conduct analyses that addressed relevant outstanding questions in the field of HIV drug resistance and which the EuroSIDA cohort study, the primary source of my data, was particularly suited to answer. An outline of my thesis structure, together with the rationale and objectives for each analysis is provided below.

Chapter 1: Background

My first chapter is a comprehensive background chapter covering the epidemiology and biology of HIV. It also outlines the basic principles of how drug resistance develops, how it is measured and the impact of both acquired and transmitted drug resistance on clinical outcomes.

Chapter 2: Methodology and Data Sources

The methodology chapter covers the strategy I used to conduct my literature reviews, which I conducted for each results chapter to describe the existing research relevant to each specific objective. I also describe the data sources and statistical methods used.

Chapter 3: Patterns of Resistance Testing and Detected Drug Resistance in Europe

The aim of this chapter was to describe the prevalence of resistance testing and detected drug resistance to the three main drug classes over time in Europe. Data on both the utilisation of resistance testing in clinical care and on the prevalence of resistance are of importance in order to plan public health activities, and EuroSIDA is an ideal setting to study resistance prevalence across Europe due to the standardized nature of the data collection and the inclusion of several Eastern European countries in the cohort, which provides data from a region that's typically not well researched.

Chapter 4: Long-term virological outcomes and resistance patterns among treatment experienced HIV patients receiving raltegravir

Whereas Chapter 3 focuses on resistance to the three major drug classes, the focus of Chapter 4 was on resistance to a commonly used drug, raltegravir, from a more novel drug class, integrase inhibitors. My aim was to describe the long-term risk of virological failure (VF) among individuals receiving raltegravir and to describe the prevalence of resistance to integrase inhibitors among those who experienced VF to this drug. As genotyping for integrase resistance is still not done routinely in clinical care in all EuroSIDA countries, I selected stored plasma samples from individuals failing raltegravir and sent them for genotyping using next generation sequencing at our collaborating laboratory in Badalona, Spain.

Chapter 5: The effect of drug resistance mutations on CD4 cell decline in HIV positive individuals maintained on a failing treatment regimen

The aim of Chapter 5 was to describe the effect of acquired drug resistance on CD4 decline among HIV infected individuals maintained on a failing regimen. Individuals who have exhausted their treatment options, today mainly those receiving care in low income settings, are sometimes kept on a failing regimen for extended periods of time. It has been hypothesised that preserving certain mutations that have a strong impact on the fitness of HIV may lead to clinical benefits for these patients, but this hypothesis has never been tested in a large cohort. In order to ensure that I had reasonable power to study the effect of individual mutations, I used data from the UK Collaborative HIV Cohort (CHIC) and the UK HIV Drug Resistance Database (HDRD) in addition to data from EuroSIDA, making this one of the largest analyses attempting to investigate this question to date.

Chapter 6: Rate of accumulation of drug resistance mutations during virological failure according to the level of viral replication

One of the primary concerns of keeping individuals on a failing treatment regimen is the risk of further accumulation of resistance. In this chapter I estimate the rate of accumulation of drug resistance among individuals maintained on a failing treatment regimen, and focus on describing how the level of viral replication affects the speed at which resistance develops. The relationship between viral exposure levels and resistance accumulation has not yet been well characterised, particularly not at low viral loads. EuroSIDA is an ideal cohort to address this question due to the relatively large availability of repeat resistance tests, arising primarily from retrospective genotyping carried out on stored plasma samples.

Chapter 7: The effect of primary drug resistance on CD4 cell decline and viral load set point in HIV positive individuals before the start of ART

My final results chapter describes the effect of drug resistance on CD4 count decline before an individual starts antiretroviral therapy (ART), as well as its effect on virulence as indicated by the viral set point. It has been hypothesised that resistance present before the start of ART might affect these factors, and therefore impact the evolution and spread of HIV on a population level. Given the potential for TDR to increase as ART is rolled-out more widely in low-income settings, an increased understanding of how the epidemic is influenced by drug resistance is important. In order to investigate this question, I used data from a large collaboration of European HIV clinics which was initiated under the ViroLAB umbrella as well as data available in EuroSIDA.

Chapter 8: Discussion and Concluding Remarks

Chapter 8 draws together the research findings from each of my results chapters, discusses the limitations of this PhD thesis and describes the implications of my findings for clinical care and future research.

1.2. History of HIV/AIDS

1.2.1. The beginning of an epidemic

The first cases of acquired immune-deficiency syndrome (AIDS) were diagnosed in 1981, when a cluster of a relatively rare and normally asymptomatic pneumonia, Pneumocystis Carinii Pneumonia (PCP), was identified among young gay men in Los Angeles (1). Soon afterwards, similar clusters of PCP and a rare infection-related cancer, Kaposis Sarcoma (KS), were identified among gay men in New York and San Fransisco (2,3). The cases had an astonishingly high mortality rate given the normally asymptomatic nature of the infections in immunocompetent individuals (4), and the underlying cause of these rare conditions was later found to be a severe immunodeficiency of an unknown cause (4). As the syndrome appeared to only affect gay men it was initially referred to in ways linking it to homosexuality, such as "gay compromise syndrome" in the medical community (5) and "gay-related immunodeficiency syndrome" in the popular press (6,7). Considerable stigma surrounded the disease at this time, and gay men were seen as the group exclusively at risk of the disease while at the same time posing a risk to the general community (8). However, cases of the disease were rapidly identified in both transfusion patients and among heterosexual individuals, particularly immigrants from Haiti in the US and people who inject drugs (9–12). In 1982 the CDC named the syndrome AIDS, defining this as the occurrence of a disease likely to be caused by diminished cell-mediated immunity, such as PCP or KS, where no other reason for immunodeficiency could be identified (13).

The epidemiology of AIDS suggested an infectious cause, and in 1983 Luc Montaignier's team at the Pasteur Institute isolated a novel retrovirus from a patient suffering from lymphadenopathy, an AIDS-defining illness. Their findings were published in *Science,* and the virus was named LAV, Lymphadenopathy Associated Virus (14). The isolation of this virus followed a number of recent scientific advances in retrovirology, including the discovery of interleukin II by Robert Gallo's lab at the National Institute of Health (NIH) , which allowed T-cells to be grown in-vitro (15). In 1984, Gallo et al published a paper which also described a novel retrovirus, HTLV-III, isolated from T-cells of 48 patients with AIDS or at risk of developing AIDS (16). Both LAV and HTLV-III were suggested as possible causal agents of AIDS, although it quickly transpired that both papers described the same virus (17). This sparked a lengthy controversy between the Paris and NIH teams, with allegations of scientific misconduct as it transpired that Montagnier had sent his first isolate of LAV to Gallo's lab before Gallo's first publication. However, Gallo was cleared from all suspicions of misconduct in 1994, and the

likely chain of events involves a contamination event at the Pasteur Institute (18). The virus was eventually renamed the Human Immunodeficiency Virus (HIV) by the International Committee on the Taxonomy of Viruses in 1986 (19), although the conflict between Gallo and Montagnier was not fully resolved until an agreement naming both the researchers "co-discoverers" was jointly endorsed by US president Ronald Reagan and the French Prime Minister Jacques Chirac in March 1987 (18,20).

In 1985 a retrovirus related to but distinct from HIV was isolated from AIDS patients in West Africa. This virus was named HIV-2, and the virus isolated from US and European patients became known as HIV-1 (21). The discoveries spurred a wave of intense research into the molecular biology of HIV, and the first diagnostic test and antiretroviral drugs were licensed in 1985 and 1987, respectively (22).

1.2.2. Origin of the HIV virus

Although AIDS was first recognized in the 1980s, the earliest cases are thought to have occurred considerably earlier (23). Medical records indicate that the first case of AIDS, retrospectively diagnosed, occurred in 1959 in Leopoldville, now Kinshasa in the Democratic Republic of Congo (24). However, modern phylogenetic techniques have estimated that the first HIV infection is likely to have occurred even earlier than this, probably in the early 20th century (25). Although estimates vary, a recent study has predicted that the first infection with the main genetic HIV strain (HIV-1 M) is likely to have occurred in 1921 (95% Confidence Interval [CI]=1908-1933) (26).

HIV-1 was introduced into the human population through zoonosis, cross-species transmission, of the Simian Immunodeficiency Virus (SIV), an asymptomatic infection found in non-human primates (23). The virus giving rise to HIV-1 has been traced back to SIV strains common in chimpanzees (27). HIV-2 is also the result of SIV cross-species transmission, but from sootey mangabeys (28). It is currently believed that the zoonosis occurred as a result of the hunting and consumption of bush-meat (25). Bush-meat hunting is traditional and a wide-spread practice in central and western Africa, as indicated by the fact that the prevalence of SIV antibodies in villages where bush meat is hunted has been estimated at 7.8% (29). This makes it likely that isolated cases of SIV infection in humans occurred before the early 1900s, and it is not known why these isolated viral infections did not turn into epidemics. It is possible that the rapidly increasing population density and sudden socio-economic changes occurring as a consequence of the colonial expansion provided the conditions required for the infection to spread (25,30), but factors such as changes in sexual behaviour, increasing STD prevalence and

circumcision practices could also have played a role (31). After entering the human population in Africa, the virus spread to a number of different regions in the world while continuously genetically diversifying (32). The introduction into the US population occurred via Haiti, potentially as a consequence of migration or the sex tourism industry (33). The introduction of the virus that gave rise to the majority of HIV circulating in the US today has been traced back to a single transmission event, as indicated by the relatively low level of genetic diversity present in the US HIV epidemic today (33). This transmission event is likely to have occurred in the late 1960s, making it possible that HIV circulated for several years in the general population before entering the high-risk gay community, where it could infect enough people in a short enough time window to make it noticeable (33,34).

1.3. Transmission

HIV can be transmitted sexually, vertically or by contact with infected blood (35). Because of this, the main groups at risk of acquiring the infection are individuals engaging in unprotected sex, people who inject drugs (PWID) and children born to mothers living with HIV. Transmission through blood transfusions is also possible, and haemophiliacs were a key risk group in the beginning of the epidemic (36).The sexual transmission risk group is commonly separated into heterosexuals and men who have sex with men (MSM) (37). This is partly because the prevalence of HIV in the MSM community is higher, which increases the probability of becoming infected during unprotected sex (37), and partly due to the fact that the risk of HIV transmission is higher during unprotected anal sex, which is more commonly practised in the MSM community (38). Despite these risk groups often being treated as separate, individuals can share several of the risk factors.

The infectivity for different routes of transmission and the measures developed to prevent new infections are described in more detail below. Infectivity is quantified as the probability of becoming infected during one potentially infectious act (39). It should be interpreted as a context-dependent measure that is ultimately determined by a combination of host, agent and environmental factors (40). Furthermore, although infectivity refers to the transmission probability per act of risk behaviour, sexual transmission estimates are sometimes reported per partner rather than per act (41).

1.3.1. Sexual transmission

The majority of new HIV infections world-wide occur due to sexual transmission (35), and it is estimated that around 80% of these infections are the result of sexual transmission among people who identify as heterosexual (42,43).

1.3.1.1. Vaginal sex

The risk of acquiring HIV through vaginal sex can differ depending on if transmission occurs from male to female or female to male, and estimates are complicated by the fact that anal sex is a contributing (often under-reported) risk factor also among heterosexuals (39,41,44). Looking explicitly at studies that reported male to female infectivity for vaginal sex, Boily et al conducted a systematic review and meta-analysis that reported a pooled infectivity estimate of 0.076% per sexual act (41). The overall infectivity estimate for heterosexual sex is somewhat higher: 0.18% per act across all study estimates, 0.38% female-to-male and 0.12% male-to-female (41). This is in agreement with an earlier systematic review by Powers et al, which found that the majority of transmission studies conducted in stable, heterosexual relationships where the prevalence of concurrent risk factors was rare, estimated transmission probabilities of around 1 event per 1000 acts (39). However, both reviews found considerable heterogeneity among study estimates, with factors such as study setting, commercial sex work and the prevalence of other STI's being important determinants of the infectivity estimates(39,41).

1.3.1.2. Anal sex

The risk of HIV acquisition is higher during anal sex than vaginal sex (38). This increased transmission probability is thought to be due to the fact that the rectal mucosa is more sensitive and easier to damage than the vaginal mucosa, and that it does not have the same humoral immune barriers found in vaginal secretions (44). A meta-analysis by Baggaley et al estimated that the transmission probability per act of unprotected anal sex is 1.4% (0.2-2.5%) (45). Interestingly, this risk was the same among both MSM and heterosexuals. The authors further found that the probability of transmission was higher for the receptive partner than the insertive partner (45), consistent with findings from a previous study from the US (46).

1.3.1.3. Oral sex

The risk of HIV infection during oral sex is harder to study, as oral sex is rarely reported as a single exposure with no other concurrent risks (47,48). A systematic review from 2008 found that 6 out of 10 identified studies reported a transmission probability of 0% - indicating that HIV transmission through oral sex is rare. However, 4 of the studies did report transmission events, with transmission probabilities ranging from 1% to 20% depending on the type of oral sex and the number of concurrent risk factors. The risk of HIV transmission during oral sex is therefore likely to be small, but not zero (47) and increased by factors including the presence of ulcers or oral pathogens (48).

1.3.1.4. Preventing sexual transmission of HIV

One of the simplest, most effective ways to prevent the sexual transmission of HIV is by using a condom (49). A Cochrane review from 2002 has estimated that consistent condom use can result in a reduction in HIV incidence of approximately 80.2% (worst case scenario: 35.4%; best case scenario 94.2%) (50), and increasing condom access and promoting condom use has become an important aspect of HIV prevention efforts world-wide (49,51). However, factors such as inconsistent or incorrect usage can significantly reduce the effectiveness of condoms (52), and gender and social inequalities mean that some individuals are not always able to negotiate condom use with their sex partners. This is a particular issue for women and commercial sex workers (CSW), who may hold very little power over their sex partners (53,54). Vaginal microbicides that do not require partner negotiation could prove beneficial to some women for this reason, and a recent trial has found that the risk of acquiring HIV could be reduced by 39% through usage of a Tenofovir containing gel (55). However, the efficacy of a vaginal microbicide depends on its acceptability and uptake, and a another recent trial have failed to confirm a protective effect of tenofovir containing gels, possibly due to a lack of adherence (56). In addition, a microbicide cannot prevent HIV transmission in instances of rape or sexual violence, and it is clear that increasing gender equality and preventing sexual violence are crucial policies to pursue alongside strategies aiming to increase condom/microbicide use (57).

Other effective ways of preventing sexual HIV transmission include circumcision (58–60) and the use of cART (61). cART is an effective preventative tool because transmission is strongly dependent on the level of viral replication, which is lowered through cART use (62). The landmark HPTN-052 trial of this 'Treatment as Prevention' paradigm has found that among discordant heterosexual couples, early and successful cART use can lead to a 96% reduction in the HIV transmission rate (61). Recently, the results from the PARTNER study has demonstrated a similarly strong protective effect of successful cART use among discordant homosexual couples; after 2 years of follow-up not a single transmission event has been observed (63). Antiretroviral treatment can also prevent HIV transmission when used in a prophylactic manner as pre-exposure prophylaxis (PrEP) in some populations (64), and after exposure as post-exposure prophylaxis (PEP) if used within 72 hours after exposure (65).

1.3.2. Vertical transmission

In 2012, an estimated 260 000 children under 15 became infected with HIV (66). The majority of these children have acquired their infection vertically, i.e. while still a foetus or an infant

from their HIV-positive mother. Vertical transmission can occur in utero (intrauterine transmission), during delivery and labour (intrapartum transmission) or as a result of breastfeeding (postnatal transmission) (67). Breastfeeding considerably increases the risk of vertical transmission, and among both breast-feeding and non-breastfeeding populations intrapartum transmission is more common than intrauterine transmission (68). The absolute risk of a transmission event ranges from 15-40% in the absence of specific interventions (69), but this risk is influenced by a range of clinical factors. The most important risk factor for vertical transmission is the maternal plasma viral load level (70). Other factors shown to influence the risk of transmission include the viral load levels in the genital tract, anaemia, prolonged membrane rupture during labour, mode of delivery, breast feeding duration and antiretroviral treatment (71).

1.3.2.1. Preventing vertical transmission of HIV

Measures that directly address the proximal risk of mother-to-child-transmission (MTCT) include HIV testing and counselling at antenatal visits, treatment with ART, safe delivery and safe infant feeding (72). It has been known that ART reduces the risk of MTCT since 1994, when the PACT-076 trial showed that an intervention of zidovudine monotherapy given to the mother during late pregnancy, injected during delivery and given to the infant for 6 weeks after birth could reduce the risk of MTCT with two thirds (73). The introduction of this intervention led to a quick decline in the number of MTCT events in high income settings, and to a lesser extent in low income settings. Simplified ART interventions have been shown to be reasonably effective and more practical to roll-out in low income settings (69), but the WHO currently recommends that in generalized epidemics all pregnant and breastfeeding women with HIV should initiate cART and maintain it as a lifelong treatment. This is known as option B+ (72). Although initiating cART is the most effective way to prevent MTCT, formula feeding (74) or birth by elective caesarean section could also reduce the transmission risk (69).

1.3.3. Parenteral transmission

Parenteral transmission of HIV includes transmission of HIV through contaminated medical injections, injecting drug use, accidental needle-stick injuries and blood transfusion. A systematic review and meta-analysis from 2006 found extensive heterogeneity among infectivity estimates in studies of injecting drug users, with estimates ranging from 0.63%-2.4% (75). The risk of acquiring HIV after receiving a contaminated blood transfusion is considerably higher, with estimates ranging from 27-100%, and pooling these weighted by study size gives an overall infectivity estimate of 80.2% (95%CI=76.7%-83.3%) (75).

1.3.3.1. Preventing parenteral transmission of HIV

As with the other transmission routes, the risk of acquiring HIV parenterally is dependent on a range of factors, including viral load and cART. Some measures previously mentioned, including PrEP and PEP are also used to minimize the risk of parenteral transmission (76,77). In addition, strategies such as offering opioid substitution therapy (OST), clean needle exchanges and bleach to disinfect needles with can reduce the risk of acquiring HIV among injecting drug users (78). Since it became routine practice to screen blood used for transfusions for HIV, transmission events via this route have grown extremely rare in high income settings (79). In low income settings the risk of HIV infection through transfusion is higher due to operational challenges, including the lack of routine screening of donated blood (80).

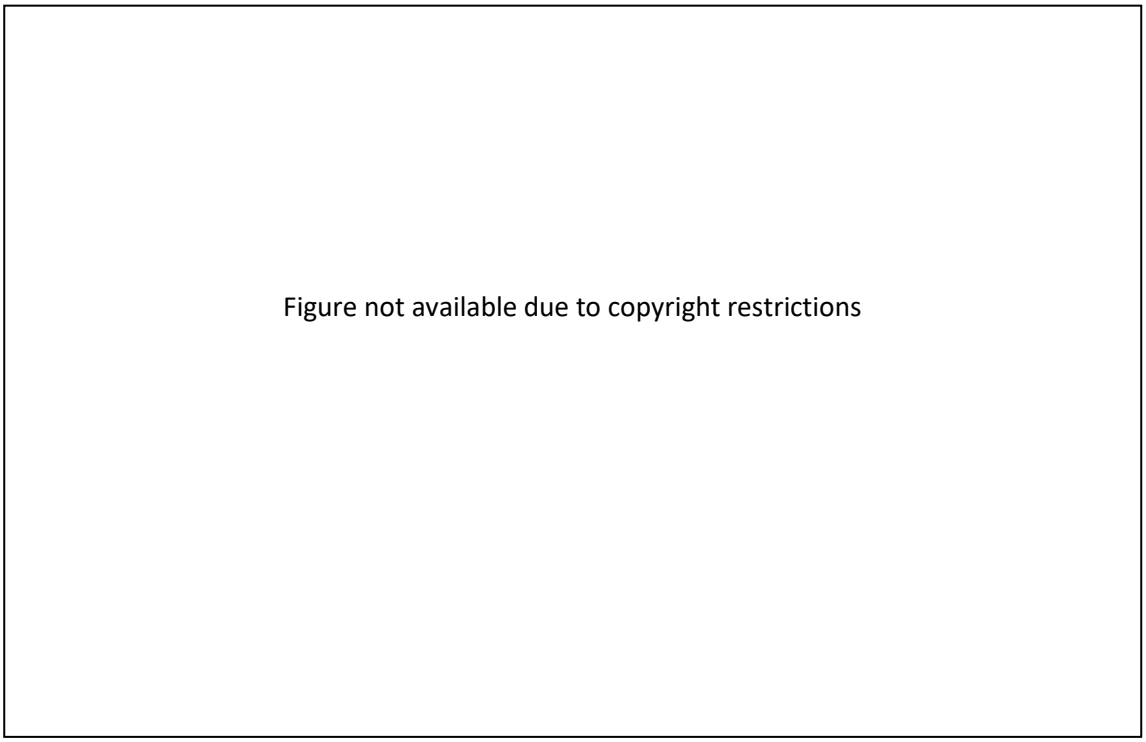
1.4. Current Burden

1.4.1. The global situation

Since the start of the epidemic, an estimated 70 million people have been infected with HIV and more than 35 million have died as a result of AIDS. In 2012, an estimated 35.3 (95%CI=32.2-38.8) million individuals were living with HIV world-wide (81).

Despite the scale of the epidemic, the incidence of HIV has started to fall on a global level. Compared to 2001, the number of new HIV infections was 33% lower in 2012, down from 3.4 (95%CI=3.1-3.7) million to 2.3 (95%CI=1.9-2.7) million (81). This has been accompanied by a reduction in the global number of annual AIDS deaths, which declined from a peak of 2.2 million in 2005 to 1.6 million in 2012. Taken together, this has caused an increase in the number of individuals living with HIV despite the concurrent fall in HIV incidence (Figure 1.1) (81).

Figure 1.1. New HIV infections, AIDS deaths and people living with HIV according to UNAIDS (2013)



Source: (81)

However, these prevalence and incidence trends vary considerably according to geographical region and transmission risk group. Sub-Saharan Africa remains the region most heavily affected by the epidemic, accounting for 68% of all people living with HIV in 2011 (Figure 1.2.) (82) and 70% of all new HIV infections (81). Heterosexual transmission is the dominant route of infection in this region. As a growing number of HIV-positive people in Sub-Saharan Africa are gaining access to HIV treatment, condoms and other interventions associated with a reduced risk of HIV transmission, HIV incidence has fallen dramatically, with an estimated 34% reduction since 2008. Remaining challenges that need to be addressed include a very high rate of TB co-infection, a lack of resources and waning political commitment (81).

Figure 1.2. Number of people living with HIV, by region

Source: (66)

1.4.2. The European situation

In Europe[1], HIV remains a public health problem despite a comparably low overall population prevalence (83). In 2013, the diagnosis rate was estimated at 15.7 per 100 000 in the EU/EEA area and Russia, and this has increased over the past decade. However, the burden of HIV is not equally distributed across the European region, with 77% of new diagnoses occurring in Eastern Europe and Russia (83). It appears that the increase in new HIV diagnoses in Europe is mainly driven by a large increase in the absolute number of newly diagnosed individuals in Eastern Europe (Figure 1.3). The number of deaths from AIDS in Eastern Europe has also increased more than 10-fold during the same period, in contrast to the decline in AIDS deaths seen globally (82).

The HIV epidemic in Eastern Europe is characterised by different key risk groups compared to countries in Western Europe (84). The main route of infection in Western and Northern European countries remains through MSM, whereas heterosexual transmission and injecting drug use are the main routes of infection in Eastern European countries, with just 2% of reported cases in Eastern Europe occurring as a result of men having sex with men (83,84). The rate of new HIV diagnoses in the region is increasing most rapidly among heterosexuals,

[1] Throughout this thesis, "Europe" or "European region" will refer to EU/EEA countries + Switzerland, Russia and Belarus, and "WHO European Region" to the WHO European region, which includes an additional 11 countries in Central Asia (http://www.who.int/about/regions/euro/en/).

whereas there is some indication that diagnoses due to reported injecting drug use is decreasing (Figure 1.4), although there is a substantial proportion of missing data on route of infection from Eastern Europe (83).

Figure 1.3. Number of new HIV diagnoses, by geographical area and year of diagnosis

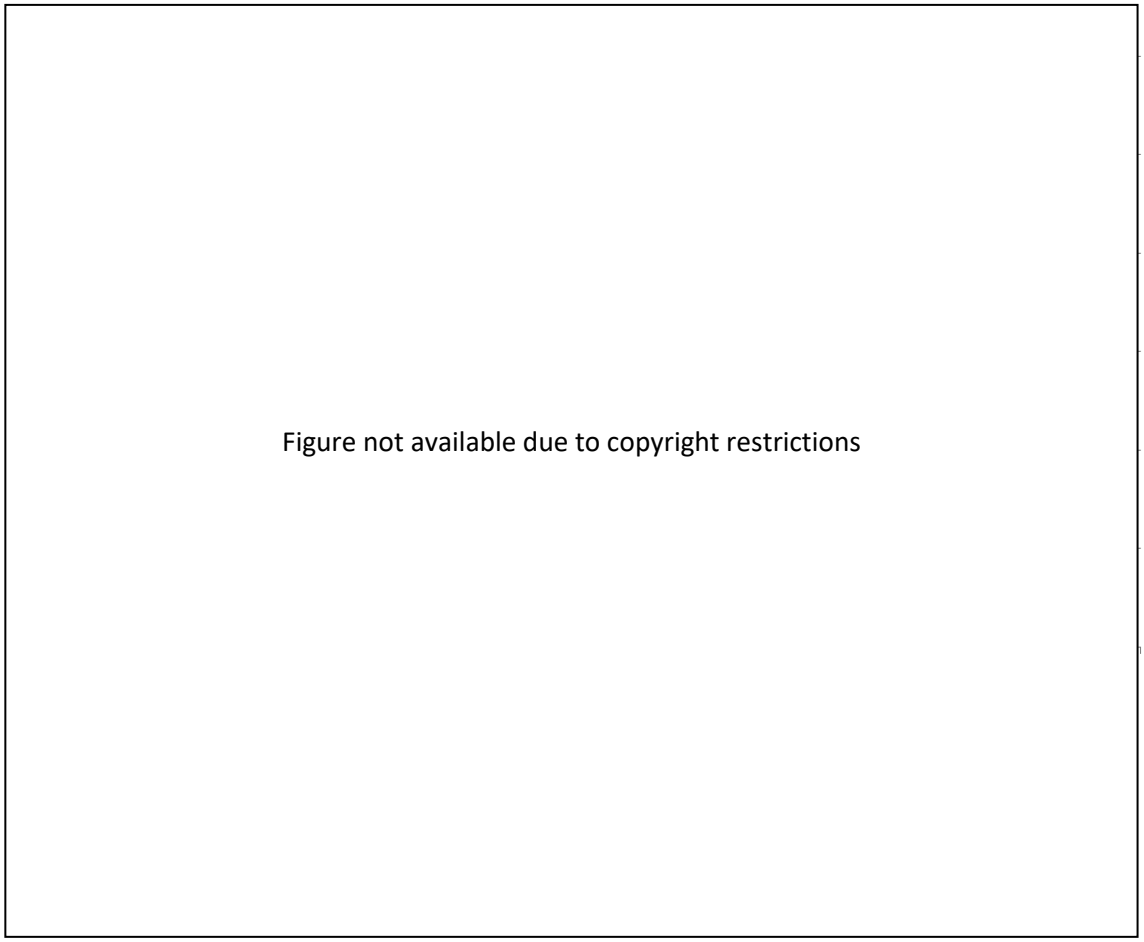
Source: (83)

Figure 1.4. Number of new HIV diagnoses, by transmission mode and year of diagnosis in Eastern Europe

Source: (83)

However, it is important to note that the situation is not homogenous across Eastern Europe, and it differs further between Central Eastern and Eastern European countries. Figure 1.5 shows the rate of new HIV diagnoses over time, plotted by country classified as 'Eastern Europe' in the WHO European Region (83)

Figure 1.5. Rates of new HIV diagnoses, by country in Eastern Europe (per 100 000)



Source: Plotted from Table 1 in (83)

Although the rate of new diagnoses is increasing in most countries, there are notable exceptions, such as Estonia and Kazakhstan. The estimated rate of new diagnoses also differ between countries, with Estonia, Russia and Moldova having notably high rates of new diagnoses (83). The increasingly problematic HIV epidemic in many countries in the Eastern Europe has its roots in the severe social and economic changes that followed the dissolution of the Soviet Union, which resulted in a fragmented healthcare system, economic turmoil and rapidly increasing alcohol and drug abuse (85). It is clear that curbing the HIV epidemic in Europe will have to include a strong focus on prevention efforts in Eastern European countries, including increasing access to harm reduction services (86). In addition, research on clinical management and outcomes in the region needs to take into account the increasing heterogeneity in both the healthcare need, organisation and delivery that exists within the post-soviet states (85,87).

1.5.Viral Biology

HIV is a retrovirus, in the family of lentiviruses (88). Retroviruses are unique within the viral classification system, as their life cycle involves the production of double-stranded DNA from RNA during the process of reverse transcription. This is the opposite of what occurs during human DNA replication, where RNA is produced from DNA during transcription (36). In order to understand antiretroviral drugs as well as drug resistance, it is important to have a thorough understanding of the molecular biology of HIV. This is outlined below.

1.5.1. Viral structure

The structure of the HIV virus can be seen in Figure 1.6 below. The genome, consisting of two copies of single-stranded RNA, is located within a protein shell called the capsid (89)**.** The HIV capsid consists of repeated protein units (p24) arranged as a cone-shaped icosahedron (90,91). The capsid serves to protect the genome from the host's immune system (89).

Figure 1.6. Structure of the HIV virus

Source: (92)

Surrounding the capsid is the envelope, a structure derived from host lipids that provides further protection for the genome. The envelope also contains viral glycoproteins, notably gp120 and gp41, that form characteristic spikes (36). The two copies of single-stranded RNA (Figure 1.7) each contain 3 structural genes (gag, pol and env), 2 regulatory genes (tat and rev) and 4 genes coding for accessory proteins (vif, vpr, nef and vpx (HIV-2) or vpu (HIV-1)) (92).

Each side of the genome is surrounded by long-terminal repeats (LTR), that contain sequences required for the start and stop of transcription (36).

Figure 1.7. The HIV-1 genome

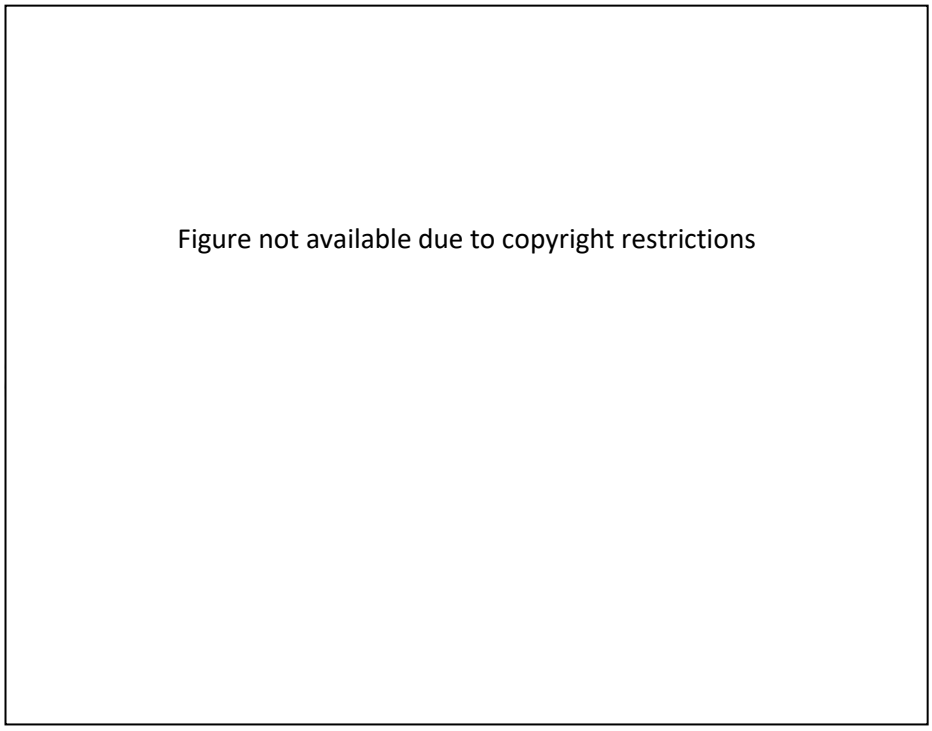
Source: (92)

The gag gene is translated into structural proteins, env into the surface binding receptors and pol contains the information needed to produce the replication enzymes, including reverse transcriptase (92). Tat and rev play key roles during HIV replication, with tat stimulating transcriptional elongation and rev involved in the transport of mRNA from the nucleus to the cytoplasm for transcription. The vif, vpr, vpu/vpx and nef genes code for, amongst other things, proteins that increase the infectivity of the virus (36).

1.5.2. Viral life-cycle

A diagrammatic representation of the HIV life cycle can be seen in Figure 1.8. Each step is described in further detail below.

Figure 1.8. The HIV life cycle



Source: (92)

1.5.2.1. Attachment and entry

HIV enters a host through mucosal surfaces or, in the case of parenteral transmission, by injection straight into the blood stream (36). It consequently infects white blood cells of the human immune system that express the surface molecule CD4 and a chemokine co-receptor. Depending on which surface receptors that are present on the viral surface, the virus infects either CXCr4 expressing T-cells (93,94) or CCr5 expressing monocytes/macrophages (95,96). The preference of the virus to infect and replicate in a certain type of cell is referred to as tropism (36). Viruses binding preferentially to the CXCr4 co-receptor are called X4 tropic, whereas viruses binding to CCr5 receptors are called R5 tropic. It is also possible for HIV to bind to both CCr5 and CXCr4. These viruses are called X4R5 or dual tropic viruses (97).

Attachment to the CD4 receptor and appropriate co-receptor triggers a conformational change in the virus, which causes the viral envelope to fuse with the cell membrane. This reveals the capsid to the intracellular environment, where its structural properties causes it to disintegrate in a process called uncoating (98).

1.5.2.2. Reverse transcription

After uncoating, the viral RNA is converted to DNA in the cytoplasm by the virally prepackaged reverse transcriptase (RT). Each virion contains between 50 and 100 RT enzymes (36). The RT

contains two enzymatic sites, an RNA and DNA-dependent DNA polymerase and an RNase H, connected by a connection domain (98). The RNA-dependent DNA polymerase reads the nucleotide sequence of the RNA genome and uses nucleotide bases present in the host cell to create a DNA-RNA hybrid helix. The RNaseH degrades the RNA strand, and the RT completes the creation of a double stranded DNA molecule. This is called the proviral DNA. The proviral DNA consequently forms a pre-integration complex together with a number of viral and host proteins. The preintegration complex crosses into the nucleus through a nuclear pore, where the integrase incorporates the proviral DNA into the host genome with the help of host enzymes (99). The resulting cell with fully integrated viral DNA is called the provirus (98).

Transcription of the DNA into mRNA occurs using host RNA dependent RNA polymerases (36). During early phases of transcription, short mRNAs encoding regulatory proteins (tat and rev) are produced. The late phase of transcription occurs once a sufficient amounts of the regulatory protein tat has been produced, and it involves the production of genomic RNA copies and longer mRNAs that retain non-coding fragments, or introns (100). These longer mRNAs are transported back into the cytoplasm through a *rev* mediated pathway, and translation occurs with the help of host cytosolic polysomes or the rough endoplasmic reticulum depending on the gene being translated (101).

Env is translated into a precursor protein for gp41 and gp120, gp160, and the gag and pol genes are translated into a precursor polyprotein for a number of viral enzymes, including the RT. Processing of precursor proteins to form the final viral produces is done by the viral protease after budding (102).

1.5.2.3. Assembly, budding and maturation

After translation, the gag polyprotein directs viral assembly. This requires that proteins and pre-cursor proteins all migrate towards the cell surface of the infected cell (102). The genomic RNA copies cluster together with pre-cursor proteins and cellular enzymes to form an immature core at the cell membrane. At the same time, surface proteins migrate and insert into the cell membrane, and capsid proteins assemble around the immature core (92,102). The immature viral particles bud from the cell surface, and thereby acquire the host-derived envelope. In order for new virus particles to be released effectively, CD4 is downregulated from the surface of the infected cell. This downregulation is caused by viral proteins nef, vpu and gp160 through a number of different processes (103–106). After successful budding and release the viral protease is activated, cleaving the precursor polyproteins to produce the integrase, reverse transcriptase and protease (102). This proteolytic cleavage of precursor

proteins is referred to as maturation, and viruses that do not undergo maturation remain as immature, non-infectious particles (36).

1.5.2.4. Latency and viral persistence

Some cells infected by HIV do not go on to produce infectious progeny (92). Instead, HIV replication is effectively put on hold while the viral genome remains integrated within the host genome. This establishment of post-integration latency allows HIV to persist within a host cell. Latent HIV has been observed in resting naïve and memory T-cells, peripheral blood monocytes/macrophages, dendritic cells and in some studies also the hematopoietic stem cells in the bone marrow. These cells are long-lived, and provide a permanent source for potential new HIV viruses (102,107,108). It is not known how HIV establishes latency, but transcriptional interference is a necessary step (109).

In addition to the long half-life of these cells, it has been suggested that HIV can persist within a host because of its ability to replicate in tissue compartments such as the central nervous system (CNS) or gut-associated lymphoid tissue (GALT) that antiviral drugs penetrate poorly. These so called sanctuary sites could also act as an HIV reservoir (110). Latency and low-level replication in sanctuary sites makes clearing HIV from an infected host a significant challenge (102).

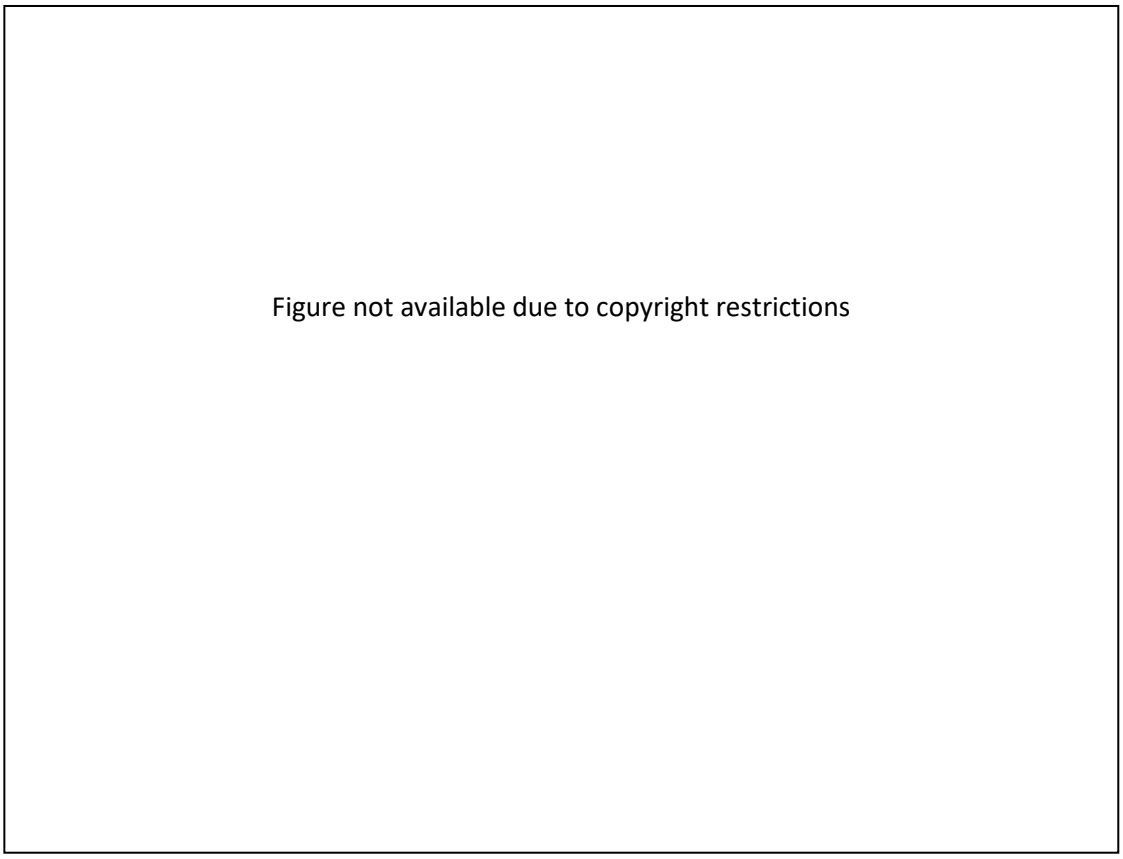
1.5.3. Genetic variability

The replication cycle of HIV is both rapid and error prone. Mutations occur at this high rate due to the RT lacking proof-reading capacity, meaning that misplaced bases cannot be excised and replaced as occurs during human DNA replication (36). As many as 10^{10} virions are produced each day in the absence of treatment, and around 1 incorrectly placed base, or mutation, is introduced per genome per replication round (92). Additional genetic variability is introduced through recombination between different viruses infecting the same host. The rate of recombination is also very high, and with an estimated 3 recombination events occurring per genome per replication cycle the recombination rate is actually higher than the mutation rate (111).

On a population level, genetic diversity can also result from the introduction of novel HIV strains into human populations. The three main genetic groups of HIV-1, M (most) , O (other) and N (new), are all believed to have arisen from independent transmission events (112). Within each of these groups there is further genetic diversity. The M genetic group contains 10 different genetic subtypes, clades, identified by letters A-K. The genetic variation between clades ranges from 25-35%, compared to 15-20% within clades (112). Recently, prevalent

recombinants of different subtypes called circulating recombinant forms (CRF's) have been identified. These arise when an individual infected with more than one subtype consequently passes on a recombinant virus (111,112). The distribution of different subtypes across regions of the world can be seen in Figure 1.9.

Figure 1.9. Global distribution of HIV subtypes



Source: (113)

Significant genetic diversity also exist on an intra-host level where so called quasispecies, viral populations containing a mix of viral genomes, are often present (36). Mutant viruses present at low frequencies, often defined as between 1 and 15% , are often referred to as minority variants (114).

The considerable genetic diversity displayed by HIV has great implications for drug design and vaccine development. The majority of drugs are developed for populations infected with subtype B, common in Western Europe and North America. In contrast, 50% of all HIV infections and 47% of all new infections occur with subtype C (112). Another important implication of the genetic variability is the rapid emergence of drug resistance in the presence

of sub-optimal drug pressure (111). This will be explored in greater detail in section 1.8 of this chapter (page 58).

1.5.4. The Immune response to HIV infection

If not treated, HIV infection ultimately leads to the depletion of CD4 expressing cells (36). It is believed that CD4 cells are killed through the cytopathic effects of HIV, but apoptosis, impaired lymphocyte regeneration and destruction of the infected cells by other branches of the immune system may also be implicated (115).

Despite being the target for HIV infection, the human immune system also displays a range of protective responses during the course of infection. Antibodies to HIV generally develop within 1-3 months following infection, and can mediate the killing of cells infected with HIV through a process known as Antibody Dependent Cellular Cytotoxicity (36). Cytotoxic T-cells (CTL's), expressing the surface molecule CD8, can also limit and supress viral replication through direct cytotoxic effects and the production of cytokines and chemokines with antiviral effects (116). The production of Interferon (IFN)-γ early on during infection restricts HIV replication and activates other parts of the immune system, while neutrophils, natural killer cells and γδ T-cells all have the ability to kill HIV infected cells through different mechanisms (117). Certain cellular restriction factors, notably APOBEC3G, TRIM5a and tetherin, are also effective inhibitors of HIV(118–120). During transcription, APOBEC3G can deaminate bases in the negative sense HIV DNA genome strand, leading to an accumulation of A->G substitutions so high that it stops the genome strand from being transcribed (121). TRIM5-a interferes with the process of uncoating, which in turn inhibits reverse transcription (119). Tetherin prevents newly synthesized virions being released from the cell by physically linking the cell plasma membrane and the virion envelope together (120,121), thus preventing the virion from maturing. A subset of individuals also have genetic defences, or genetic resistance to HIV infection. This includes individuals homozygous for the delta32- CCr5 deletion, who lack functional CCr5 co-receptors and as a result are immune to infection by R5 tropic viruses (122).

Unfortunately, HIV has evolved ways of evading the immune responses mounted against it, and host defences typically fail to control viral replication. As a consequence, the vast majority of HIV infections results in a decline in immune function (36). The stages of the infection will be outlined in greater detail in section 0 (page 45).

1.5.5. Measuring HIV: viral load and CD4 counts

The amount of HIV circulating in the blood (viral load, VL) as well as the number of CD4 cells (CD4 cell count) is used to measure the effectiveness of antiretroviral treatment and the progression of HIV infection (36). Both the VL and CD4 count have been proven to be valuable prognostic markers (123–130).

1.5.5.1. HIV viral load (VL)

The VL is measured by quantifying the number of copies of HIV per millilitre of blood plasma (copies/mL). Techniques for measuring VL can be split into technologies that rely on nucleic acid testing, such as RT-PCR, and non-nucleic acid based testing (131). RT-PCR involves the amplification of a target RNA sequence followed by its quantification (see section 1.8.5.2) (36). Non-nucleic acid based technologies quantify correlates of VL rather than measuring nucleic acid directly. This can involve measuring RT activity or the level of circulating p24 protein, and then correlating this to derive an estimate of the VL level (131). There are also point-of-care based tests that can distinguish between VLs above and below different thresholds (132,133) .

In high income settings, HIV RNA is normally quantified from blood plasma. However, it is also possible to use dried blood spots (DBS) to measure the VL (134). This is of particular use in low income settings, where clinical sites may need to send specimens to a reference laboratory in order to receive VL measurements. HIV RNA can generally be reliably quantified from DBS at high VLs, although accuracy is not as good at lower VLs, and can vary according to which platform is used (131,135).

1.5.5.2. CD4 counts

The CD4 cell count is measured as the number of CD4 cells per mm^3 of blood, although it is standard practice to also measure the CD4 cell percentage, which is proportion of all lymphocytes expressing CD4 (136). Recently, using the CD4/CD8 ratio as a clinical marker has also grown more popular, as it may capture signs of immunoactivation and immunosenescence in a more comprehensive way compared to the absolute CD4 count (137,138).

Although there are several different methods for measuring CD4 counts available, the gold standard is a technique referred to as flow cytometry (139). This involves a focused stream of cells being passed through an optic censor, with the degree of light scattering being used to estimate the CD4 proportion (140). This can consequently be used to estimate the absolute CD4 count using either a haematology analyser (dual platform) or by utilising a pre-determined blood volume, sometimes mixed with a known number of fluorescent micro-beads (single

platform) (139,140). CD4 counts can also be measured manually, by counting the number of cells using a light microscope. However, this technique is very labour-intensive, and measurements will be at least partially subjective (140).

What technology is used will differ according to the resources available. Although the WHO recommends a point of care based test, it further states that country-level leadership should determine which technology is most appropriate for a given setting (141).

1.5.5.3. Defining failure and success

The goal of HIV treatment is to prevent disease and death by ensuring that CD4 counts and immune function are preserved. However, as clinical events have grown increasingly rare it is common practice to use surrogate laboratory markers to measure the success of HIV treatment (142). This has several benefits: the outcome is easy to quantify and may be more commonly achieved than a clinical end-point. There are also some drawbacks, as a change in a biomarker may not translate into a tangible clinical benefit (143). A meta-analysis evaluating the use of VL and CD4 count as surrogate end-points has shown that both have value as prognostic markers, but both have also shown discordant effects on clinical outcomes in different trials (144). Despite these considerations, achieving an undetectable VL, that is, having viral replication supressed to such a low level that no viral RNA can be measured, is commonly used as a definition of treatment success in both clinical trials and observational studies (142,145). What is considered undetectable will depend on the detection limit of the assay used to measure the VL. Many assays now have a detection limit as low as 50 or 20 copies (cp)/ml, and therefore these cut-offs are often used to define an undetectable viVL (146) . A commonly used term to describe a lack of treatment efficacy is virological failure (VF). VF has been defined in a plethora of ways across studies, but it is commonly defined as either a rebound of the VL to detectable levels following suppression to undetectable levels, or a failure to suppress the VL to undetectable levels despite ongoing treatment. The threshold with which VF is defined varies according to the assay used.

Treatment failure can be defined either using virological or immunological measures, but virological measures is the recommended approach (72). The WHO defines virological failure as a confirmed (two measures within a three month interval, with adherence support between measures) VL of >1000 cp/ml after at least 6 months of using ART (72). In Europe, a lower threshold of VL>50 is often used to define virological failure (147).

1.5.5.4. Guidelines on monitoring

Both virological and immunological monitoring is part of standard of care in high income settings, although VL measurements are considered the gold standard for the detection of treatment failure (72). In high income settings, both VL and CD4 measurements are taken every 3-6 months or more often depending on patient need, although these recommendations are primarily based on expert opinions (146).

For low income settings, the WHO recommends that VL measures are taken 6 months after starting ART and then every 12 months. CD4 counts should ideally be done every 6 months after the start of treatment. If VL measurements cannot be done, clinical or immunological monitoring should be done to detect treatment failure (72). VL monitoring is recommended above CD4 or clinical monitoring, as it can aid an earlier detection of treatment failure, thus enabling more timely switches to second-line regimens and preventing the accumulation of drug resistance (72,148). The ideal frequency of monitoring VL monitoring is not known, and is likely to vary between different settings (149).

1.6. Natural History of HIV infection

Although the course of the disease has been drastically changed following the discovery and introduction of cART, it is still important to understand the process by which HIV can lead to disability and eventually death. Due to the lack of an effective treatment in the years following the discovery of the HIV virus, the natural history of the disease is relatively well described. Without any intervention, the HIV disease process can be broadly split into three stages: the primary stage, the asymptomatic stage and the symptomatic stage or AIDS (Figure 1.10) (150). Each stage will be described in more detail below, and the impact of treatment will be considered in greater detail in section 1.7.8 (page 58).

Figure 1.10. The different stages of HIV infection in the absence of treatment

Source: Adapted from (150)

1.6.1. Primary stage

After HIV enters the body, the virus starts replicating and the VL expands exponentially. At this stage there are no antibodies to the virus, but viral antigens such as p24 can be used to diagnose the infection (151). The rapid increase in the VL is mirrored by a decline in the CD4 counts (150,152). After 4-6 weeks antibodies towards the virus start to develop (seroconversion) (36), and during this period the individual may experience non-specific symptoms similar to those of mononucleosis or influenza, such as a fever, rash, headache, muscle aches or nausea (153–155). It has been estimated that between 25-65% of individuals experience symptoms of primary infection (152). After the development of antibodies, CD4 counts recover and the VL stabilises at a level called the viral set-point (152).

1.6.2. Asymptomatic stage

The temporary recovery of the CD4 cells marks the beginning of an asymptomatic, chronic phase of the disease process that is characterised by stable but high viral turnover (156). It is often referred to as a period of 'clinical latency', to clarify that the lack of symptomatic disease is not indicative of virological latency (156,157). In fact, viral replication is generally high during this time, but constrained by the actions of the immune system (116). For a number of reasons these immune controls eventually fail, and the early increase in CD4 count is followed by a decline (158). CD4 counts have been estimated to decline with 20-78 cells per microliter per year, although this is influenced by a range of factors, including the VL burden, age and VL

setpoint (150,158–160). Individuals normally remain symptom free as long as CD4 counts are above 350 cells per microliter (152).

1.6.3. Symptomatic stage/AIDS

After around 10 years, CD4 counts tend to have declined to a level that makes individuals susceptible to opportunistic infections (Table 1.1), but the time this takes is variable (150). In fact, between 2 and 5% of individuals can maintain CD4 counts within healthy limits for up to 20-25 years. These individuals are called long-term non-progressors (LTNP) (161). A subset of LTNP, so called elite controllers, are in addition able to supress viral replication to levels below detection (<50 cp/ml) without treatment (162).

A diagnosis of AIDS is made based on both laboratory and clinical evidence. Following a laboratory confirmed HIV diagnosis, a decline of CD4 counts to less than 200 or the presence of one of the opportunistic infections listed in Table 1.1 is commonly used to categorise an individual as suffering from AIDS (163). The survival time following an AIDS diagnosis differs depending on the presenting condition and has changed considerably over time (164,165). The median survival time in the early years of the epidemic (1984-1985) following any AIDS diagnosis has been estimated as 11.6 months, varying between 3.3 and 19.1 months depending on the opportunistic infection (165).

Table 1.1. AIDS defining conditions in laboratory confirmed HIV cases

Condition	*Notes*
Bacterial infections, multiple or recurrent	Only among children <13 years
Candidiasis of bronchi, trachea, or lungs	
Candidiasis of esophagus	Condition may be diagnosed presumptively
Cervical cancer, invasive	Only among adults and adolescents aged >13 years
Coccidioidomycosis, disseminated or extrapulmonary	
Cryptococcosis, extrapulmonary	
Cryptosporidiosis, chronic intestinal	>1 month's duration
Cytomegalovirus disease (other than liver, spleen, or nodes)	Onset at age >1 month
Cytomegalovirus retinitis (with loss of vision)	Condition may be diagnosed presumptively
Encephalopathy attributed to HIV	
Herpes simplex: chronic ulcers) or bronchitis, pneumonitis, or esophagitis	>1 month's duration, onset at age >1 month
Histoplasmosis, disseminated or extrapulmonary	
Isosporiasis, chronic intestinal	>1 month's duration
Kaposi sarcoma	Condition may be diagnosed presumptively
Lymphoid interstitial pneumonia or pulmonary lymphoid hyperplasia complex	Only among Children <13 years, condition may be diagnosed presumptively
Lymphoma, Burkitt (or equivalent term)	
Lymphoma, immunoblastic (or equivalent term)	
Lymphoma, primary, of brain	
Mycobacterium avium complex or *Mycobacterium kansasii*, disseminated or extrapulmonary	Condition may be diagnosed presumptively, only among adults and adolescents aged >13 years
Mycobacterium tuberculosis of any site, pulmonary, disseminated, or extrapulmonary	Condition may be diagnosed presumptively
Mycobacterium, other species or unidentified species, disseminated or extrapulmonary	Condition may be diagnosed presumptively
Pneumocystis jirovecii (previously known as "*Pneumocystis carinii*") pneumonia	Condition may be diagnosed presumptively
Pneumonia, recurrent	Condition may be diagnosed presumptively, only among adults and adolescents aged >13 years
Progressive multifocal leukoencephalopathy	
Salmonella septicemia, recurrent	
Toxoplasmosis of brain	Onset at age >1 month, condition may be diagnosed presumptively
Wasting syndrome attributed to HIV	

Source: Adapted from (163)

1.6.4. Factors determining disease progression

The rate of disease progression in HIV infection can vary considerably depending on a range of genetic, biological, psychosocial and structural factors. The proximal determinant of disease progression is the level of viral replication as quantified by the VL. This in turn is influenced by viral properties, but also the host immune response (36,166). Individuals infected with HIV-1

have a faster disease progression compared to individuals infected with HIV-2 (167). There is also some evidence suggesting that the rate of disease progression varies according to viral subtype, and individuals infected with a non-A non-B subtype may progress faster to AIDS than those infected with subtype A (168,169). Gender (170), older age (171) and ethnicity (172) can also affect the rate of disease progression. Since the development of cART, the main determinant of disease progression is whether a person is on antiretroviral treatment or not (173).

1.7. Antiviral Drugs

The first drug against HIV was approved by the U.S Food and Drug Administration (FDA) in 1987 (174), and since then 27 drugs have been approved by the European Medicines Agency (EMA) for the treatment of HIV in Europe, with more currently being developed (174,175). HIV drugs are divided into different classes based on what function of the virus they target. The three main drug classes are nucleoside reverse transcriptase inhibitors (NRTI) , non-nucleoside reverse transcriptase inhibitors (NNRTI) and protease inhibitors (PI) (36). However, drugs targeting the processes of viral attachment, entry, DNA integration, maturation and envelope fusion have also been developed (175–179). A comprehensive list of HIV drugs according to drug class and date of license can be found in Table 1.2 below. Drugs from different classes and even within a specific drug class can have different levels of efficacy and different genetic barriers. The genetic barrier refers to the number of mutations HIV needs to develop before acquiring resistance to a particular drug (180). Efficacy is often estimated as the proportion of people in a clinical trial that achieve an undetectable VL, although a composite end-point such as the proportion of people who do not experience VF or switch their treatment can also be used (145).

Current first line HIV therapy consists of a combination of three HIV drugs, referred to as combination antiretroviral therapy (cART) or highly active antiretroviral therapy (HAART). These combinations consists of two NRTI drugs (referred to as the backbone) and either an NNRTI, PI or more recently an Integrase Inhibitor (II) (146,147). All HIV drugs can cause a number of side-effects, ranging from mild (such as headache and rash) to severe or even life-threatening (such as lactic acidosis, kidney failure andincreased suicidality) (181). As side-effects depend on the mode of action of the drug they tend to be shared across a particular drug class, but some individual drugs may have particularly beneficial or risky side-effect profiles. The drug classes, together with their mode of action and current use are outlined in more detailed below.

Table 1.2. Drugs licensed for the treatment of HIV infection

Generic Name	Brand Name (Company)	Date of License	Pivotal Studies	Current Use (EACS guidelines)
NRTI				
Zidovudine (AZT)	Retrovir (ViiV)	FDA approval[1]: 1987	(182), (183)	Considered in specific circumstances (such as pregnancy or TB-coinfection)
Didanosine (ddI)	Videx (Bristol Meyers Squibb)	FDA approval[1]: 1991	(184), (185)	No longer recommended for use.
Zalcitabine (ddC)	Hivid (Roche)	FDA approval[1]: 1992	(186), (187), (188)	No longer recommended for use.
Stavudine (d4T)	Zerit (Bristol Meyers Squibb)	1996	(189)	No longer recommended for use.
Lamivudine (3TC)	Epivir (ViiV)	1996	(190), (191)	1st line NRTI backbone (with Abacavir)[5]
Abacavir (ABC)	Ziagen (ViiV)	1999	(192), (193)	1st line NRTI backbone (with Lamivudine)[4]
Tenofovir (TDF)	Viread (Gilead)	2002	(194)	1st line NRTI backbone (with Emtricitabine)[6]
Emtricitabine (FTC)	Emtriva (Gilead)	2003	(195), (196)	1st line NRTI backbone (with Tenofovir)
NNRTI				
Nevirapine (NVP)	Viramune (Boehringer	1998	(197), (198)	2nd/ 3rd line agent
Efavirenz (EFV)	Sustiva (Bristol Meyers	1999	(199)	1st line 3rd agent[7]
Etravirine (ETV)	Intelence (Janssen-Cilag)	2008	(200), (201)	2nd/ 3rd line agent
Rilpivirine (RPV)	Edurant (Janssen-Cilag)	2011	(202), (203)	1st line 3rd agent[8]
Delaviridine		Not approved in Europe		No longer recommended for use
PI				
Indinavir (IDV)	Crixivan (Merck Sharp &	1996	(204), (205)	No longer recommended for use
Ritonavir (RTV)	Norvir (AbbVie)	1996	(206)	Used with other PI's as a booster
Saquinavir (SQV)	Invirase (Roche)	1996	(207), (208)	Option in certain circumstances, for example serodiscordant couples who wish to conceive

Nelfinavir (NFV)	Viracept (Roche)	1998	(209)	License not renewed for European market
Amprenavir (AMP)	Agenerase (GSK)	2000	(210)	License not renewed for European market
Lopinavir (LPV)[2]	Kaletra (AbbVie)	2001	(211)	1st line 3rd agent
Fos(-amprenavir)	Telzir (ViiV)	2004	(212), (213),	2nd/3rd line 3rd agent
Atazanavir (ATA)	Reyataz (BMS)	2004	(215), (216),	1st line 3rd agent
Tipranavir (TPV)	Aptivus (Boehringer Ingelheim	2005	(218), (219)	2nd/3rd line 3rd agent
Darunavir (DRV)	Prezista (Janssen-Cilag)	2007	(220), (221),	1st line 3rd agent
Entry/Fusion				
T-20/Enfuvirtide	Fuzeon (Roche)	2003	(224), (225),	2nd/3rd line 3rd agent
Maraviroc (MVC)[3]	Celsentri (ViiV)	2007	(226), (227)	2nd/3rd line 3rd agent[9]
Integrase inhibitors				
Raltegravir (RGV)	Isentress (Merck Sharpe and Dohme Limited)	2007	(228), (229), (177)	1st line 3rd agent
Elvitegravir (EGV)	Vitekta (Gilead)	2013	(230)	1st line 3rd agent
Dolutegravir (DGV)	Tivicay (ViiV)	2014	(231), (232),	1st line 3rd agent

1.Brought to European market before EMA was founded.
2.As coformulation with ritonavir.
3.Only licensed for treatment experienced individuals
4.Contraindicated if HLA B*5701 positive, should be used with caution if high predicted CV risk
5.Should not be used if HBsAg positive
6. Avoid TDF if osteoporosis, renal monitoring required
7. Not to be given if history of suicide attempts or mental illness, not active against HIV-1 or HIV-1 group O
8. If VL <100,000 cp/ml and CD4 counts > 200 cells/mm^3
9. Only if CCr5 tropic virus

Source: compiled and adapted from (234), (235), (174),(146) and (147)

1.7.1. NRTI

NRTIs are structural analogues of the cellular nucleosides/nucleotides used by the HIV RT to construct viral DNA (236). There are currently (2012) 8 licensed NRTIs, all with a similar mechanism of action (237). After absorption, the drug is phosphorylated by cellular kinases in order to convert it into its active form. The resulting substrate is consequently incorporated into the growing DNA chain by the HIV RT, where it terminates chain elongation and thus inhibits viral replication due to the lack of an hydroxyl group required for further bases to be attached (237,238). Zidovudine, or AZT, was the first compound to be licensed for the treatment of HIV infection following trials showing that it could reduce short-term mortality among individuals with AIDS (182). Despite this initial success, zidovudine is rarely used today because of its side-effects (146,183) , although it may occasionally be prescribed for individual patients with reduced number of drug options or pregnant women (147). Several alternative NRTIs were developed shortly after zidovudine, but many are no longer recommended for use because of their associated toxicities (146). Current recommendations in high income settings list tenofovir and emtricitabine as the most suitable NRTI backbone in first line HIV treatment, with abacavir and lamivudine as alternatives (72,146,147).

Many of the side-effects of NRTIs have been linked to mitochondrial dysfunction induced by the drugs. Such side effects include potentially fatal liver damage, myopathy, cardiotoxicity and peripheral neuropathy and lipodystrophy; a syndrome of localised fat redistribution where wasting occurs on the face, legs, arms and buttocks and fat accumulation occurs around the abdomen, breasts and neck (147,239,240). Evidence from the Data Collection on Adverse Events of Anti-HIV Drugs (D:A:D) collaboration has suggested that abacavir is associated with an increased risk of myocardial infarction (MI) (241), and tenofovir has been associated with an increased risk of both acute and chronic nephrotoxicity (242). A systematic review of prospective studies has confirmed the association between tenofovir and kidney dysfunction, although the magnitude of the effect when pooled across studies is modest from a clinical perspective, and may not warrant changes in first line therapy in settings where kidney monitoring is feasible (243). The association between abacavir and MI has not been reproduced in a large FDA meta-analysis of clinical trials (244).

1.7.2. NNRTI

NNRTIs are group of diverse molecules that induce and bind to a hydrophobic pocket near the active site in the RT (237,245). Binding of the NNRTI induces a conformational change in the RT active site, which disrupts its normal function and inhibits viral replication (236). The first compounds discovered that could inhibit the RT in this way were discovered through two

separate processes: as a result of chance (HEPT derivatives, (246)) and following a large programme of rational drug screening (TIBO derivatives, (247)). This allowed for the rational design and discovery of several other compounds that shared a similar mode of action. However, despite over 50 molecules discovered that can be classed as NNRTIs (248), only four (efavirenz, etravirine, rilpivirine and nevirapine) have made it to the European market (234,237,249). All of these are effective only against HIV-1, as HIV-2 has structural differences that renders it intrinsically resistant to NNRTIs (250).

The current WHO guidelines recommend that efavirenz is used as the third agent in first line HIV therapy (72), even though concerns are starting to be raised regarding the potential harm of CNS side-effects that have been linked to the drug (251). Next-generation NNRTIs, such as rilpivirine, have a slightly better side-effect profile and a higher genetic barrier against resistance (252), but also slightly more complex dietary requirements (146). All NNRTIs can cause a number of non-specific side-effects, the most common one being a fairly mild and self-limiting rash (253).

1.7.3. PI

PIs differ from NRTI and NNRTIs in that their main target is the viral protease rather than the RT (237).They were discovered through rational drug design in 1990, and there are currently 10 different licensed PIs (indinavir, ritonavir, nelpinavir, fos-amprenavir, atazanavir, tripanavir, lopinavir, saquinavir, lopinavir, darunavir) (234,254). PIs contain a motif that mimics the proteolytic area of the viral protein that the protease is normally required to cleave. However, as the drug itself cannot be cleaved it instead binds to and inhibits the protease, thereby preventing newly produced virus from maturing (236,255). Recently, it has been suggested that in addition to preventing maturation, PIs also affect viral entry, reverse transcription and post-transcription events, although the mechanisms behind these activities are not yet fully elucidated (256). The first licensed PI inhibitor, indinavir, was brought to market in 1996, and it was quickly followed by the development of ritonavir (234). Both these drugs are no longer used in clinical practice because of limited potency as well as toxicity issues, however, ritonavir instead used at low doses to boost the bioavailability of co-administered PIs (257,258), as it is a potent inhibitor of the cP450 metabolic pathway (259). Current guidelines recommend that a PI is used as a third agent in the case of VF, although depending on patient and clinician preference it could also be used as part of first line therapy (146).

Mild side-effects are common among patients receiving PIs and include non-specific symptoms such as nausea and diarrhoea (234). More severe side-effects include hyperglycaemia and

lipodystrophy, which has been reported as more common for PIs compared to NRTIs (240). Recently developed drugs, such as Darunavir, have been associated with less severe side effects (260).

1.7.4. Entry inhibitors/CCr5 antagonists

Two currently approved drugs, enfuvirtide and maraviroc, interfere with the ability of HIV to enter into new cells (237). Enfuvirtide is a peptide that prevents the fusion of the viral and cell membrane by interacting with a domain in the gp41 protein (261). Maraviroc is a small molecule that binds to the CCr5 co-receptor, thereby preventing its interactions with the viral gp120 protein (96). Because it binds to the CCr5 co-receptor, maraviroc is only effective in individuals with an R5 tropic virus (176).

Enfuvirtide is not commonly used, as its chemical structure means it is not orally bioavailable. Instead, it needs to administered through subcutaneous injection twice a day (236). The need for an injection is associated with side-effects, including localised injection-site reactions (262). Although enfuvirtide can still be an option for patients with few remaining drug options, its side effects, low efficacy and low genetic barrier limits its clinical usefulness (262,263).

1.7.5. Integrase inhibitors

The final class of drugs currently licensed for the treatment of HIV infection are integrase strand transfer inhibitors (INSTIs), or integrase inhibitors (II), which target the viral integrase (236). Their mode of action involves inhibiting the process of strand transfer, through which viral DNA becomes attached to cellular DNA (264). There are currently three licensed II's that all seem to be well-tolerated and potent: raltegravir, elvitegravir and dolutegravir (236). Current guidelines recommend integrase inhibitors as one possible option as a third agent for starting antiretroviral therapy, and dolutegravir in particular has been shown to be effective when used as salvage therapy, even among patients who previously failed raltegravir (146,265).

The side effect profile of INSTIs are favourable, likely due at least in part to the lack of a cellular equivalent of the integrase (266). However, they can still cause nausea, headache and diarrhoea. The use of dolutegravir has additionally been associated with sleep disturbances (266).

1.7.6. Novel drug developments

New treatments for HIV are continuously being developed. Recently, the results from a phase IIb trial of a new attachment inhibitor, BMS-663068 or fostemavir, were published (179).

Fostemavir interferes with the ability of HIV-1 gp120 to bind to the CD4 receptor, and it has been shown to have comparable efficacy to boosted atazanavir with a similar side effect profile (179). The drug has recently entered into phase III trials in treatment experienced individuals (267). A number of maturation inhibitors that target the gag polyprotein, thereby preventing the cleavage event that is necessary for the production of mature virions, have recently been developed (268). BMS-955176 showed similar efficacy to boosted atazanavir, and is currently undergoing phase 2b dose-finding studies (269). Although monoclonal antibodies were found to be ineffective for the treatment of HIV in early clinical trials, antibodies produced using more modern single-cell based cloning methods appear to have good potential for supressing viral replication in animal models as well as in humans (270). The 3BNC117 antibody is currently being evaluated for safety and antiretroviral activity in Phase I clinical trials (271).

Developments of drugs in the existing drug classes are also occurring. Doravirine, a next generation NNRTI, has a different resistance profile compared to older NNRTIs, and has recently completed phase IIb studies with promising results (272). New formulations of older drugs are also being brought to the market. A long-acting formulation of rilpivirine that can be given by monthly injections rather than orally, of particular use for patients with adherence problems, has recently been developed (273). In order to address concerns over kidney toxicity, a new formulation of tenofovir as tenofovir alafenamide, TAF, has recently been licensed. TAF has been shown to be non-inferior in virological efficacy compare to tenofovir (274), but has a better safety profile for renal and bone health (275). There are also on-going investigations looking at optimal dosing strategies for already approved drugs. The ENCORE study compared 400 mg efavirenz to the standard 600 mg dose, and found that the reduced dose was non-inferior to the 600 mg dose, but with a better side effect profile (276).

There has also been a recent focus on developing treatment strategies and drugs that could potentially cure HIV. One such group of treatments are the histone deacetylase (HDAC) inhibitors (277). HDAC inhibitors operate by preventing the de-acetylation of histones associated with viral DNA. This can cause latent HIV to reactivate (278). Combined with effective cART, the aim of HDAC containing therapy is to eradicate the viral reservoirs through a ‘shock and kill’ strategy (279). Although in-vitro studies of this approach have been promising, the effectiveness of HDACs in the clinic is still uncertain (280) .

1.7.7. Treatment strategies

In the early 90s HIV treatment consisted of only one drug (monotherapy) due to the limited number of drugs available. However, it was quickly realised that patients receiving zidovudine alone would develop resistance to the drug (281). A formal evaluation of dual-therapy for HIV infection occurred in 1995 in the ACTG 175 study (282). In this trial, comparing zidovudine alone, zidovudine + didanosine, zidovudine + zalcitabine and didanosine alone, Hammer et al found that dual therapy was more effective than zidovudine alone and equally effective as didanosine alone among patients with no previous ART exposure (282). This was confirmed a year later in the DELTA trial (283). However, despite the important effects these medications had on short-term outcomes or surrogate markers, it quickly transpired that dual therapy was not effective in patients with advanced HIV disease (284), patients who had received prior zidovudine monotherapy (285) or for the prevention of long-term mortality (286). The development of the first PIs opened up the for combination therapy based on two different classes of drugs, and the evaluation of triple therapy for treating HIV followed shortly after this in 1996 (207). In a trial published in the New England Journal of Medicine, Collier et al compared a combination regimen of saquinavir, zidovudine and zalcitabine to dual regimens of zidovudine and either saquinavir and zalcitabine for patients who had previously received zidovudine for at least 4 months. The triple-combination arm was more effective in reducing viral replication and increasing CD4 counts than were either of the dual therapy arms (207). This was later confirmed for ART-experienced individuals in the MRK 035 study (287) and for treatment-naïve individuals in the INCAS study (198). Regimens consisting of four different drugs have also been compared to triple-therapies in large trials, but not found to be superior to triple therapy (288), although a recent small proof-of-concept study found that initial therapy with four drugs may be slightly more beneficial for individuals with a baseline VL >100,000 cp/ml (289).

The large number of drugs licensed to treat HIV has raised questions about which combination of drugs is the most effective, and a plethora of trials attempting to answer this question have been undertaken (290). Potential first line treatment combinations according to different guidelines can be seen in Table 1.3 (291). There are a number of recent studies that have compared the efficacy of different first line combinations. The ACTG 5257 study found that a regimen of two NRTIs + raltegravir was superior to a regimen of two NRTIs and a boosted PI (292). The SINGLE study found that abacavir/lamivudine + dolutegravir was superior to a tenofovir/emtricitabine/efavirenz regimen (293), and the FLAMINGO trial found that a combination of 2 NRTI + dolutegravir outperformed a two NRTI + boosted PI regimen (294).

The current treatment recommendations are based on a combination of the efficacy of the regimen, cost and availability (72,146), and the ideal regimen is likely to also differ according to patient preference (295).

Treatment guidelines are continuously evolving, and recent studies have cast some doubt on whether triple combination therapy is still necessary now that more potent drugs have been developed. The GARDEL trial evaluated dual therapy of a boosted protease inhibitor (lopinavir) and one NRTI versus standard cART of lopinavir and two NRTIs. At 48 weeks, patients on dual therapy did not have significantly worse outcomes than patients on triple therapy (296). These findings have been confirmed for lopinavir in the OLE trial (297) and for atazanavir in the SALT trial (298). Monotherapy with protease inhibitors has been shown to be non-inferior for HIV maintenance therapy (299), and is likely to be more cost-effective than triple therapy, even if it were to lead to a higher rate of virological failure (300). Preliminary studies have also reported surprisingly good outcomes for patients on dolutegravir monotherapy (301) or dual therapy of dolutegravir and lamivudine (302).

Table 1.3. Recommended first line cART regimen according to different guidelines

	NRTI	**NNRTI**	**PI**	**II**
US DHHS 2015 (303)	TDF/FTC ABC/3TC	--	DRV/r	DTG, EVG, RAL
IAS-USA 2014 (304)	TDF/FTC ABC/3TC	EFV/RPV	ATV/r DRV/r	DTG, EVG, RAL
EACS 2014 (147)	TDF/FTC ABC/3TC	EFV/RPV	ATV/r DRV/r	DTG, EVG, RAL
WHO 2014 (72)	TDF+3TC or FTC	EFV	-	-
BHIVA 2015 (146)	TDF/FTC	RPV	ATV/r DRV/r	EVG RAL

Source: Adapted from (291)

Traditionally, when to start ART has been determined by CD4 counts. The first HIV treatment guidelines, presented at the International AIDS Society (IAS) conference in Vancouver 1996, stated that individuals with a CD4 count below 200, or who presented with a CD4 count between 200-500 and with symptoms of AIDS should initiate zidovudine monotherapy; all other individuals were recommended no therapy (305). This has evolved considerably. In 2015, results from the START trial were published, which showed that starting treatment immediately irrespective of CD4 count, rather than waiting until it decreased to below 350, as per guidelines at the time, was associated with a lower risk of clinical events (306). This was

also confirmed in the TEMPRANO study of early antiretroviral therapy and isoniazid preventative therapy in Cote d'Ivore (307). Most guidelines, including the WHO, have now been updated and recommends that individuals diagnosed with HIV should start treatment immediately (72,146,147).

1.7.8. HIV in the post-cART era

With effective treatment, HIV positive individuals in high income settings now have a life-expectancy that is approaching that of the general population (308,309). However, even though effective cART has transformed the way that HIV is managed, the infection and its treatment can still cause a number of unwanted complications, including an increase in the co-morbidities associated with ageing (310). Studies suggest that HIV infected individuals are at an increased risk of non-AIDS related events, including heart disease, liver dysfunction, kidney disease, osteoporosis, neurocognitive impairment and non-AIDS related cancers compared to the general population: a risk only partly explained by the presence of traditional behavioural risk factors in some studies (311). This has given rise to a hypothesis of accelerated immunsenescence. Immunosenescence is the process by which the immune system declines in function as a result of natural ageing (310). In HIV, the presence of chronic low-level inflammation and immune activation may cause this process to accelerate (312). Current research is generally supportive of this hypothesis, and biomarkers indicative of inflammation has been linked to an increased risk of cardiovascular disease (313), cancer (314) and neurocognitive impairment (315). However, some studies show contrasting results. A Danish cohort found that the excess risk of morbidity and mortality seen in HIV positive individuals were mainly due to risk factors that we present before the start of cART, and that survival in patients on cART without the presence of traditional risk factors was almost identical to that in the general population (316). In addition, there is no trial showing a clear benefit of reducing the excess inflammation caused by HIV (317). As the HIV positive population continues to age, the interest in the areas of chronic immune activation, inflammation and co-morbidities in the management of HIV is likely to continue to grow (318).

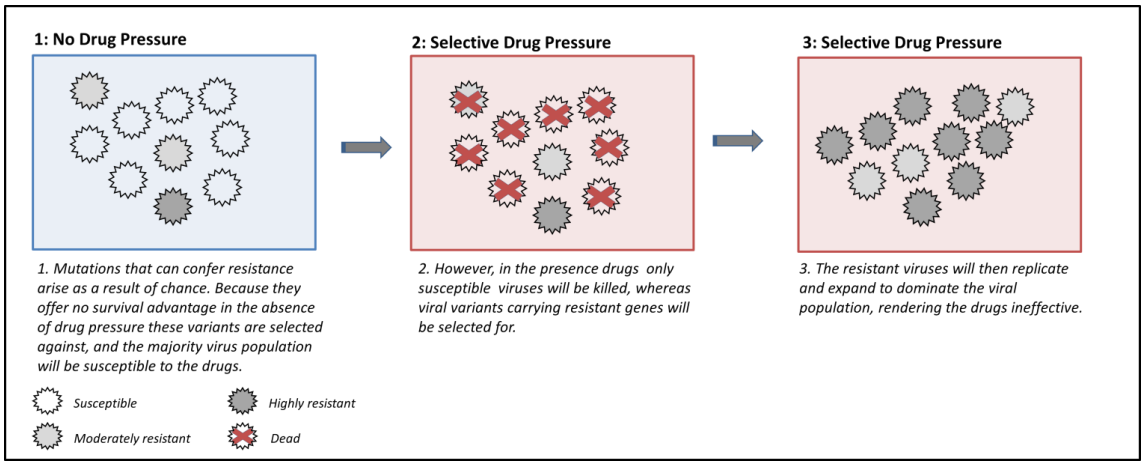
1.8. Drug Resistance

1.8.1. Principles of resistance

Despite the success of combination therapy in prolonging the survival of HIV-infected individuals, resistance to one or more of the drugs in a regimen can develop (36,319). Drug resistance is defined as changes to a microorganism that cause a reduction in drug susceptibility (320). For HIV, this is primarily caused through mutations in the viral genome

that emerge as a consequence of the very high replication rate of the reverse transcriptase, its poor proof-reading capacity and the high rate of genetic recombination (described in detail in section 1.5.3, page 40). Due to natural selection (321), resistant viruses will be selected for survival in the presence of selective drug pressure over strains that do not carry resistance mutations. The resistant strains will eventually expand to dominate the existing viral population (36,322). An illustration of this process can be seen in Figure 1.11.

Figure 1.11. Selection of drug resistance

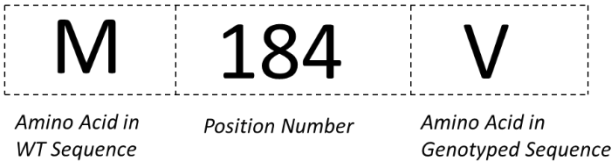


When resistance develops during treatment as a consequence of selective drug pressure, it is referred to as secondary or acquired resistance. This is in contrast to primary or transmitted resistance, which is present before the start of antiretroviral treatment. Escape mutations refer to mutations in the HIV genome that allow the virus to evade the response of the host's immune system (323). Such mutations often can develop before the start of treatment and include mutations that change the HIV epitope; the pattern of surface antigens that make the virus recognisable to host's immune system (323).

Most mutations that confer resistance are caused by single base (nucleotide) substitutions, but duplications and insertions can also lead to resistance (322). As there are many combinations of nucleotides that can code for the same amino-acid, a given base change does not necessarily change the amino-acid. For example, the amino acid arginine (Arg, A) is encoded through both the codon "AGU" and "AGC". A change in the 3rd position of such a triplet from 'U' to 'C' would not result in a change in the amino-acids. Such nucleotide changes are referred to as silent mutations.

In HIV drug resistance, all mutations are described according to the same standardised system, which involves numbering the amino-acid residues in the different HIV genes. The residue number will be preceded by a letter indicating the wild-type (WT)sequence amino-acid, and followed by a letter indicating the amino-acid identified in the sequence under study (Figure 1.12). If the amino acid in the studied sequence is different from that in a reference sequence, a mutation is considered to be present. It is common practice to use the sequence of a subtype B laboratory strain (HXB2 or NL43) or a subtype B consensus sequence summarising the most common amino-acid residues at different positions as the reference strain, as there is no clearly defined HIV WT sequence due to the high genetic variability of the virus (324).

Figure 1.12. Illustration of genotypic data for HIV



In the absence of selective pressure, viral strains carrying resistance mutations often disappear from the majority circulating virus. This occurs at different speeds depending on a range of factors, including the fitness cost of particular mutations (325,326) and the number of back-mutations required for resistance to reverse (327). Nonetheless, strains carrying particular resistance mutations can persist as archived genomes in latently infected cells, and can rapidly emerge once a drug regimen is re-initiated (328).

1.8.1. Viral fitness and replicative capacity

The amount of time it takes for resistance to develop and be selected for will depend both on the prevalence of a mutation, the amount of drugs present, and the selective advantage conferred by the mutation (322). Selective advantage is defined as the traits of an organism that allows it to stay alive and reproduce to a greater extent than other organisms in a given environment (321). This is a concept closely linked to the fitness of the virus, which refers to the ability of a virus to replicate and spread in a given environment. When a mutation appears, this is often associated with a reduction in the fitness. Some mutations, called compensatory mutations, can however enhance viral survival by restoring viral fitness while not influencing the susceptibility to a particular drug (329). Viral fitness is often approximated by measuring the viral replicative capacity (RC) (330). The RC refers to the average number of cells that are infected by one typical virus-infected cell. In practice, this is measured through comparing how well a particular viral isolate replicates in comparison to a WT reference strain. A variety of

assays that quantify viral replication in different ways can be used for this purpose, such as specific infectivity assays, single-cycle replication capacity assays, parallel culture assays and competitive growth virus co-culture (331). The RC is reported as a proportion or a percentage, and it's calculated using the level of replication of the reference strain as the denominator (332). This makes the RC of the reference strain equal to 1 (or 100%). Values for the RC less than 1 (or 100%) imply impaired RC and fitness of the mutant strain (332). The theoretical definition of RC implicitly includes the effect of the human immune system on the viral replication rates. However, in-vitro assays cannot take this restrictive effect of the immune system into account, and estimates of RC from assays should be interpreted with this in mind (330). Fitness can also be characterised using what is called a fitness landscape, which describes the fitness of the virus as a mathematical function of its amino acid sequence (Figure 1.13) (333). The benefit of using a fitness landscape to quantify the impact of mutations on a genome is that it explicitly takes interactions between mutations into account. Interactions between mutations, where the presence of one mutation either enhances or supresses the effect of another mutation, are called epistatic interactions.

Figure 1.13. Illustration of fitness landscape

Source: (333)

1.8.2. Mechanisms of drug resistance

Resistance emerges in different ways depending on the drugs taken. The main mechanisms that can lead to resistance to each of the main drug classes are outlined briefly below.

1.8.2.1. NRTIs

Resistance to NRTIs mainly develop by two different mechanisms. Firstly, the appearance of certain mutations can allow the RT to discriminate between NRTIs and the cellular nucleotides used to construct DNA, thereby avoiding the incorporation of the drug into the growing DNA strand (319). Mutations that cause resistance in this way include M184V/I, K65R, 70E/G, L74V, Y115F and the relatively rare Q151M complex (319,334,335). In many populations the most common of these mutations are M184V/I, which alone is enough to confer high-level resistance to lamivudine and emtricitabine (335,336)).

Secondly, the NRTI can be removed from the DNA chain through ATP (adenosine triphosphate)-mediated phosphorolysis after incorporation. The thymidine analogue resistance mutations (TAMs) confers resistance through this mechanism (334,337). They are referred to as TAMs as they are primarily selected for by zidovudine and stavudine, which are both structural analogues of the cellular nucleotide thymidine (337). The TAMs tend to develop according to two characteristic patterns: TAM 1 (41L, 210W, 215Y) and TAM 2 (67N, 70R and 219E/Q) (338,339). There is also evidence that mutations can arise as a result of APOBEC3G/F induced diversification (section 1.5.4, page 42). The inhibition of APOBEC3G/F activity by the viral protein vif is in some cases only partial, which can lead to levels of genetic diversity that are not lethal (340). Such diversity can actually result in the development of mutations that confer drug resistance, including resistance to lamivudine (341).

1.8.2.2. NNRTI

Resistance to NNRTIs develops relatively easily, and most of the mutations to these drugs affect the binding of the NNRTI to its target hydrophobic pocket in the RT (319). This can involve mutations causing either alterations to the size of the binding pocket or physical changes to the structure of the binding pocket that prevent drug access (334). A single mutation can be enough to confer resistance to several of NNRTIs (342). The L1001 mutation confers resistance to efavirenz, rilpivirine and nevirapine and the K103N mutation is commonly found among individuals failing efavirenz (50% of patients) and nevirapine (30% of patients) (343). Newer generation NNRTIs such as etravirine and rilpivirine have a higher genetic barrier than the older drugs, although resistance to these drugs can still develop. Mutations E138K and M184I have been associated with high-level resistance to both etravirine and rilpivirine (344).

1.8.2.3. PI

Development of resistance to PIs is complex, and for ritonavir boosted PIs it requires the development of at least two mutations (one or more major and one or more compensatory) in the protease gene (319). It can also involve mutations in the gag polyprotein (345). Major mutations in the protease gene affect the binding of the protease to its polyprotein target (335). Compensatory PI mutations are often referred to as minor PI mutations, and are very common (346). They can improve viral fitness, but in the absence of major PI resistance mutations they have little effect on treatment outcomes (346). Compensatory mutations include mutations in the cleavage site of the gag polyprotein, which increases the ability of the protease to bind to this substrate, leading to more efficient production of viral progeny (345).

Boosted PIs have a higher genetic barrier than NRTIs and NNRTIs, at least partly due to the fact that the development of resistance requires more than a single mutation to arise. This has led to the suggestion that VF on PIs primarily occurs in people who do not adhere to their treatment, rather than as a result of drug resistance (319). However, it could also be that the mutations associated with protease resistance have not yet been fully characterised or that resistance occurs in other regions of the HIV genome than the protease. Recently, mutations in the env gene that encodes the HIV envelope have been reported to be associated with resistance to PIs (256), but how common these mutations are found among patients failing PIs remain to be elucidated (347).

1.8.2.4. Entry inhibitors

Resistance to maraviroc, a CCr5 receptor antagonist, can occur through a shift in co-receptor usage by the virus or through changes in the viral envelope that allows for binding to the CCr5 receptor despite the presence of the inhibitor (348,349). Changes in co-receptor usage mainly occurs if there is a mix of R5 and X4 tropic viruses present at the start of therapy (319), and existing data indicate that the changes in the viral envelope is a more common cause of maraviroc resistance (348). Resistance to the fusion inhibitor enfuviritide can develop through mutations in a domain of 10 amino acids (36-45) in the gp41 gene (335,349).

1.8.2.5. Integrase inhibitors

First generation integrase inhibitors raltegravir and elvitegravir have a relatively low genetic barrier compared to protease inhibitors, and a single point mutation (such as T66K, E92Q, G188R, F121T or N155H/S/T) can confer resistance to either of these drugs (342). However, the exact mechanisms by which resistance arises are still unclear (350). It has been suggested that mutations that have been associated with resistance affect the flexibility of the integrase

(350), and an increased rate of drug dissociation could also be implicated (351). Dolutegravir has a higher genetic barrier to resistance, and it has been shown to be effective at suppressing VL levels even among patients who have previously failed raltegravir (265). Although rare, resistance to dolutegravir has been observed among patients pre-exposed to integrase inhibitors (352), but not among patients starting the drug from naïve (353).

1.8.3. Acquired drug resistance

The prevalence of acquired resistance after VF varies, and estimates range from 76%-90% among viraemic individuals tested for resistance (354). Individuals more likely to acquire resistance include those who receive suboptimal regimens (180) and those who are poorly adherent (355). The prevalence and risk factors for acquired resistance will be investigated and discussed in more detail chapter 3.

1.8.3.1. Clinical implications of acquired resistance

The presence of drug resistance limits the number of available treatment options (356,357), and individuals with drug resistant viruses are typically forced to rely on either complex multi-drug regimens, older more toxic treatments or newer, more expensive drugs in order to supress viral replication (180). Some individuals can develop extensive drug resistance to all three major drug classes (triple-class resistance; TCR) (358). Although TCR is rare nowadays, estimated at 3% of all treatment experienced patients, it is complex to the compose a regimen that fully controls viral replication for these cases (358,359). A further negative consequence is the potential for individuals who have acquired drug resistance to transmit viruses with mutations causing resistance, which in turn can compromise the use of standard first-line regimens (360).

The effect of acquired drug resistance on markers of clinical progression in the presence of continued treatment remains partially undocumented, and depends both on the extent and type of resistance present. Some studies have shown that individuals with acquired drug resistance can preserve CD4 counts despite VF in the presence of continued treatment (357,361,362). Furthermore, a study by Sigaloff et al has indicated that individuals failing first line therapy with drug resistance are not more likely to experience consequent VF, even if the second-line regimen is predicted to have reduced effectiveness (363). It is possible that some drugs are still able to exert residual activity despite the presence of resistance (332), and the fitness cost of certain mutations could also limit the effect they have on clinical progression (364). However, several large cohorts have shown that the emergence of drug resistance during therapy is associated with increased mortality and morbidity (365,366), particularly

among individuals with triple-class resistance (367–369). The effect of acquired drug resistance on immunological prognostic markers will be investigated and further described in chapter 6.

1.8.4. Transmitted drug resistance (TDR)

Transmitted drug resistance is present in about 10% of new infections in Europe, and the proportion of new infections with TDR in Europe is stable (370) and possibly decreasing in the some countries (such as the UK) (371). Risk factors for being infected with a resistant viral strain include Caucasian race and potentially viral subtype, although this is likely to be at least in part explained by the higher availability of cART in certain geographical areas (372).

1.8.4.1. Clinical implications of TDR

TDR has been shown to increase the risk of VF unless genotypic testing is conducted to construct a fully suppressive regimen (373,374). The presence of TDR has also been shown to increase the rate of CD4 decline before the start of treatment in the first year of following infection (375), and is predicted to have a significant effect on mortality outcomes on a population level (376). The effect of TDR on clinical progression before the start of treatment will be investigated and described in chapter 7.

As mentioned in section 1.8.1, viruses with resistant mutations tend to be less fit than drug-susceptible viruses, and therefore transmission of resistance occurs less often than what would be expected given the potential pool of transmitters (377). The lower fitness of drug resistant variants also means that viruses carrying resistance mutations may gradually be outcompeted by non-resistant variants in the absence of treatment (360), although some studies have indicated that TDR mutants can persist for longer than previously thought despite the lack of drug pressure (378). This is believed to occur when the infection is established by a relatively homogenous population of resistant viruses, which may require multiple reversions in order for a drug-susceptible variant to arise (378) or because of the decreased fitness of different mutants (379,380). Irrespective of the persistence of TDR in the majority circulating virus, resistant variants may still remain archived within a host due to the establishment of latency, and the composition of the initial regimen for a patient with TDR should take this into account (360,381,382).

1.8.5. Testing for drug resistance

In order to guide the choice of antiretroviral drugs, resistance testing can be performed. This can be done in two different ways; as genotypic resistance testing or phenotypic resistance testing (319). Genotypic resistance testing involves determining the order of the nucleotides, sequencing, in the genome of the virus, whereas phenotypic testing involves measuring how

quickly the growth of a viral strain can be inhibited by different drug concentrations (383). These techniques will be outlined in more detail below.

1.8.5.1. Phenotypic resistance testing

Phenotypic resistance testing involves growing a recombinant viral strain in cell culture in the presence of different dilutions of the drug of interest (319). This allows the degree of viral replication to be measured and plotted for each drug concentration. The resulting sigmoid dose response curve can be used to find the concentration of drug that inhibits 50% of viral replication. This value is called the inhibitory coefficient (IC) 50, although the IC90, the concentration at which 90% of drug replication is inhibited is sometimes also reported (319). In order to ease the interpretation of these results, they are often reported as "fold-resistance", in which the degree of viral inhibition is calculated by comparing the IC50 of the resistant virus with the IC50 from a wild-type or consensus virus (354).

Figure 1.14. Result of phenotypic resistance test (IC50)

Source: (384)

The main benefit of phenotypic testing is that complex resistance patterns can be interpreted relatively easily, as the cumulative effect of all mutations are reported. On the other hand the procedure is slower and more expensive compared to genotypic resistance testing (385). There are currently two main phenotypic resistance assays, Antivirogram (Virco) and PhenoSense (ViroLogic) (386), although neither is commonly used as part of current clinical care in Europe.

1.8.5.2. Genotypic resistance testing

Genotyping resistance testing involves sequencing specific genes or gene regions, normally the protease and RT (383). All sequencing methods require a certain amount of DNA in order to work effectively, and the HIV genome is therefore converted to DNA and amplified before it is sequenced (387). Amplification commonly occurs through polymerase chain reaction (PCR). Briefly, amplification occurs by placing the DNA sample together with key enzymes and buffers in a thermal cycler. Samples are then heated, which causes the DNA to denature, or separate. The temperature is then lowered slightly, which triggers the amplification of a target section of each DNA strand. This process is then repeated a large (>20) number of times. Once a sufficient number of copies of the target sections of the genome have been generated, sequencing can take place (387). Classic Sanger sequencing using the dideoxy chain-termination method follows a principle similar to PCR, but instead of adding substrates (dNTP) to the samples that allow copies to be made, a mixture of cellular dNTPs and ddNTP, which terminates chain elongation due to the lack of an 3' OH group, are added (388). This means that the polymerization process generates a number of partial copies of the original strand, all of a different length. These are consequently separated through gel-electrophoresis, and fluorescent labels are used to identify the terminating ddNTP in each position. Specialised software can consequently translate this into a DNA sequence that represents the average sequence present in the sample of the PCR products (387). The two main Sanger sequencing platforms in use are the Trugene HIV-1 assay (Visible Genetics Inc.) and the Viroseq assay (Abbott Laboratories) (383). These normally require that the VL in the original plasma sample contain at least 1000 copies of the virus per ml for optimal performance, although sequencing at VLs between 500-1000 can also be achieved (383). Ultrasensitive sequencing using different protocols can enable genotyping at VLs even lower than this (389).

Recently, so called next-generation sequencing (NGS) technologies have been developed. Although the term covers a wide variety of technologies using different protocols, all of them parallelise the sequencing process, which means that many different DNA strands can be processed simultaneously (390). The end-result gives you a read-out of each sequence present in the sample, rather than an average as in Sanger sequencing (391), as is illustrated in Figure 1.15 below. This means that the data generated can be used to study minority variants that could not be described using Sanger methods, and is the reason why it is sometimes referred to as 'ultra-deep' sequencing. Ultra-wide sequencing refers to the sequencing of the entire length of the viral genome (392). NGS is still come at a relatively high cost, and the large amount of data generated also make data analysis time-consuming, and requires

bioinformatics expertise (390). This currently limits the use of NGS technologies in clinical practice. There are also mutation-specific assays that only assess the presence of a given mutation in a specific position (392). These could be of particular use in low income settings (393).

Irrespective of the method, the final result of a genotypic resistance test is a description of the RNA sequence or sequences, which is compared to a reference HIV strain to generate a list of mutations (324). Mutations considered indicative of drug resistance will generally have been identified through in vitro passage experiments, susceptibility testing of laboratory or clinical isolates, sequencing of individuals who are failing a particular drug and/or studies of the association between baseline genotype and consequent virological response (335).

Figure 1.15. Output from different types of sequencing

Source: (392)

A mutation list can be interpreted in different way using different interpretative tools:

1) Lists of mutations that are considered associated with each drug, such as the IAS-US list (338)

2) Expert based genotypic interpretation systems that assign a "score", often referred to as a genotypic sensitivity score (GSS), to each drug depending on which mutations are present and the degree of resistance they are considered to confer. These systems include the ANRS score system, the Stanford Resistance database, HIV-GRADE and the AntiRetroScan system (342,394–396).

3) Web-based bio-informatics tools that can predict treatment response based on a number of factors, including resistance. These include EU-Resist and RDI HIV-TrePS (397,398).

Comparisons of the different interpretation systems have found reasonable agreement between them (399,400), and all predict virological outcomes (401). A final way of interpreting genotypic data is to relate it to phenotypic susceptibility, for example using the Geno2Pheno or Virtual Phenotype platform (402). There are also tools that use results from both genotypic and phenotypic tests to generate an interpretation (e.g. Phenosense GT) (403).

1.8.5.3. Guidelines for resistance testing

Resistance testing is recommended in high income settings by all major guidelines both before the start of therapy, ideally as soon after infection as possible, and upon treatment failure (146,147). At present, most guidelines recommend genotypic over phenotypic testing at least for initial resistance testing, as it tends to be quicker, cheaper and can detect resistance that is still evolving (319). Several randomized controlled trials have shown a benefit from genotypic resistance testing to guide therapy switches after VF, but trials evaluating phenotypic resistance testing have failed to show a benefit as compared to either no resistance testing or genotypic testing alone (Table 1.4). Nonetheless, phenotypic resistance testing may still have some value for patients who have experienced multiple failures and therefore may have complex resistance patterns (354,383).

When interpreting the evidence underlying these policy recommendations, it is important to take into account that the majority of these trials were done at a time when a higher proportion of patients had been exposed to mono or dual therapy before starting their first cART regimen and at a time when future drug options were limited (404). The changing clinical context of HIV care is likely to influence the utility of resistance testing (385), and a 2004 review of trials of resistance testing has found that the overall clinical benefit from resistance testing may be relatively small (404). Clinicians are likely to take into account factors such as the patients adherence, technological availability, cost and the number of available treatment options in addition to the advice provided by guidelines when deciding whether or not to do a resistance test (385), and the utility of the test will depend both on previous drug history of the patient and the number of available treatment options. The use of resistance testing in clinical practice will be investigated and described in chapter 3.

Table 1.4. Clinical trials evaluating the utility of resistance testing following virological failure

Trial (Reference)	Year	Comparison	N	Population	Result
Viradapt (405)	1999	Genotype v Standard of Care (SOC)	65 v 43	Patients in whom therapy was not successful (a VL of >10,000 after at least 6 months' treatment with a protease inhibitor)	Genotypic resistance testing resulted in a significantly better virological response at 6 months
GART (406)	2000	Genotype + Expert Advice (EA) v SOC	78 v 75	Patients with a threefold or greater rise in plasma HIV-RNA on at least 16 weeks of combination therapy	Genotypic resistance testing gave a better virological response over 12 weeks.
ARGENTA (407)	2002	Genotype v SOC	85 v 89	At least 2 month of HAART and a VL of greater than 2000 cp/ml on two consecutive determinations or less than 1 log reduction of RNA more than 2 months after the start of the last regimen	Genotypic resistance testing led to a better virological response at 3 months but not at 6 months compared to SOC.
Havana[1] (408)	2002	Genotype v SOC EA v SOC	207 v 119 164 v 162	Patients on stable cART who presented with VF (HIV RNA>1000 cp/ml) after being on a particular regimen for more than 6 months	Genotypic resistance testing showed benefit at 12 weeks compared to SOC in the main ITT analyses; EA showed benefit in a sub-group analysis or in per-protocol analyses only.
VIRA3001 (409)	2002	Phenotype v SOC	142 v 130	Patients who failed to achieve or maintain virological suppression (HIV-1 RNA plasma level >2000 cp/ml) after taking triple therapy for at least 1 month.	No evidence that phenotypic resistance testing provided virological benefit at 16 weeks; some evidence of benefit in secondary analyses.

CCTG 575 (410)	2001	Phenotype v SOC	Total 256[2]	Patients with 6 months of previous ART, exposure to no more than two prior PI's and failure of the current regimen (HIV RNA?400 cp/ml) and a stable regimen (no change at least 4 weeks prior to screening)	Virological response was not different at 6 months or 12 months.
Narval (411)	2003	Genotype or phenotype v SOC	183 v 183 v 152	HIV plasma RNA >1000 cp/ml, previous exposure to at least one PI for 3 months, unchanged antiretroviral regimen for the 2 preceding months.	Genotypic resistance testing was associated with improved virological response at 12 weeks compared to SOC in multivariate
CERT (412)	2004	Access to genotype v Access to phenotype v SOC	151 v 152 v 146	Patients who received stable ART containing at least 2 drugs for 8 weeks before randomization	No benefit of either genotypic or phenotypic resistance testing on long-term outcomes over SOC.
RealVirFen (413)	2003	Phenotype v Virtual Phenotype	139 v 137	HIV infected adults failing their current ART (RNA >1000 cp/ml)	No difference between phenotype and virtual phenotype.
ERA (414)	2005	Genotype v Phenotype and Genotype	152 v 159	Patients who had swapped ART, with a most recent RNA measurement above 2000 cp/ml and the clinician could not select a potent regimen of 3 or more drugs without access to a resistance test	No added benefit of phenotypic resistance testing in terms of virological response at 12 months.
CREST (415)	2006	Genotype v Genotype + Virtual Phenotype	170 v 168	Patients with an RNA >2000 who wanted to change therapy	No added benefit of virtual phenotype in terms of virological response at 48 weeks.

1.8.6. Preventing drug resistance

The development of drug resistance can be prevented in several different ways. As mentioned previously, the benefit of using cART is the rapid suppression of viral replication to such low levels that development of resistance is prevented. This means that in the context of cART, acquired resistance is often the consequence and not the cause of VF (322). Adherence is a key factor that affects the likelihood of a person experiencing VF, and is also strongly linked to the development of drug resistance (416). The relationship between adherence and resistance development is not straight-forward, and it likely differs depending on the drug class (Figure 1.16). Single PI resistance is most likely to develop at moderate to high levels of adherence, resistance to ritonavir boosted PIs at moderate levels of adherence and NNRTI resistance at low to moderate levels of adherence (417). Very low adherence is likely to lead to a removal of selective drug pressure, which would prevent the development of resistance.

Figure 1.16. Resistance development as a consequence of adherence

Source: (417)

There are several ways adherence can be improved. For example, it has been shown that individuals who have a greater understanding of the disease process and resistance development are more likely to show good adherence (418). Counselling and patient support programs could also improve adherence and thereby prevent resistance development (419). The use of regimens with a higher genetic barrier, such as boosted PI based cART or dolutegravir based cART, could prevent the development of resistance compared to use of NNRTI cART which has a low genetic barrier compared to these options. Regimens which encourage good adherence, such as those with a relatively low pill burden and no complex dosing requirements may also prevent the development of resistance (420). Finally, addressing structural factors, including stigma, could improve individuals adherence to ART (421). There are also programmatic actions that can be taken to prevent the development of drug

resistance. This includes ensuring frequent VL monitoring, improving the supply of drugs to prevent stock outs and the removal of barriers for accessing continued treatment, such as financial or travel-related barriers (422,423). Continuous surveillance of both acquired and transmitted drug resistance is also important in order to inform policy that can ensure that levels of drug resistance do not compromise the response to the HIV epidemic.

Chapter 2 . Methodology and Data Sources

2.1. Literature Reviews

Literature reviews were conducted through PubMed for each chapter (424) . The questions and search terms used to identify relevant articles are shown below in Table 2.1. Each search was built up using medical subject headings (MeSH) terms. For topics where MeSH headings were not available, I used the PubMed option “all fields” to search for that term. For each search, the same search strategy was applied (Figure 2.1).

Figure 2.1. Search strategy

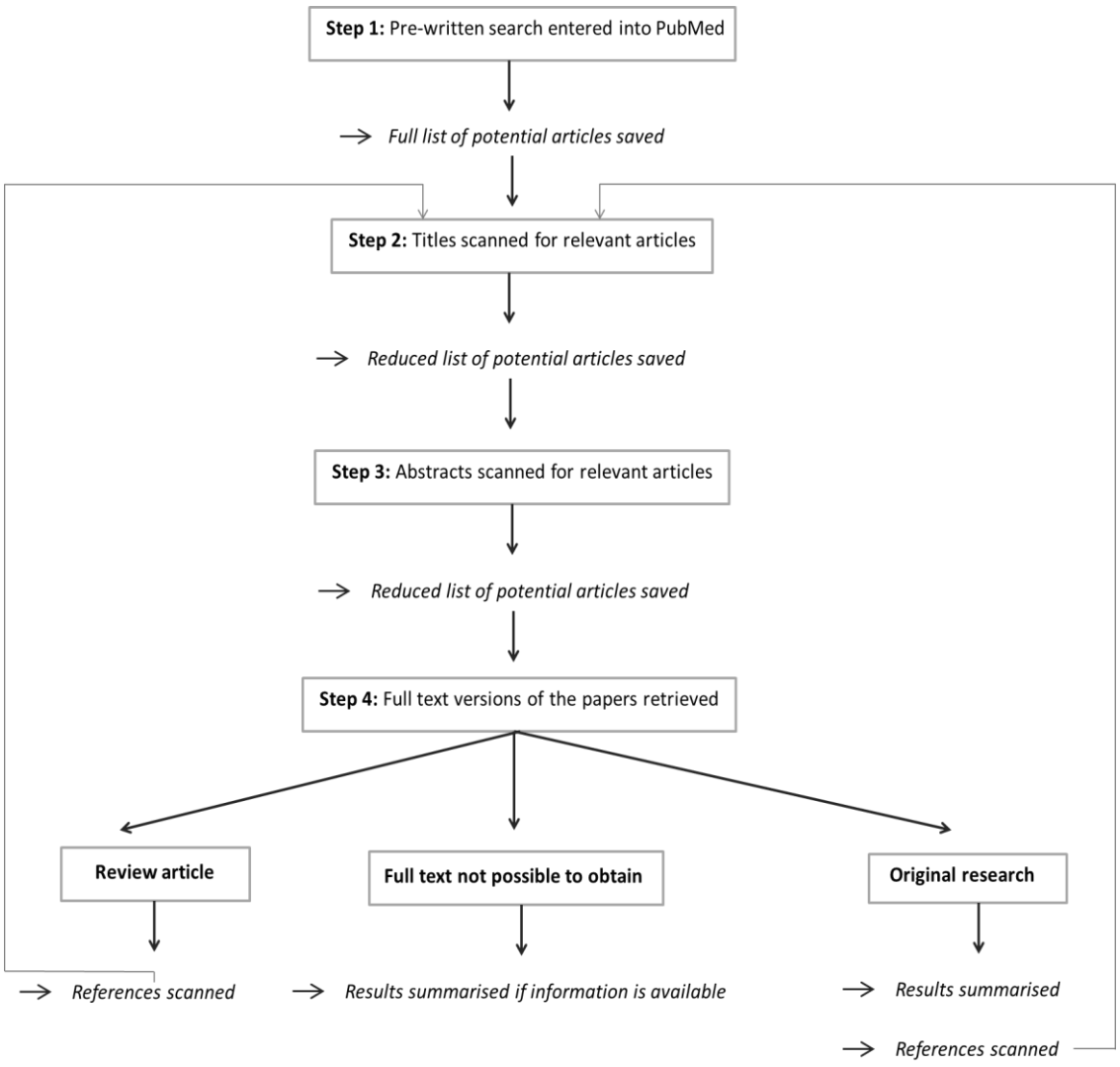


Table 2.1. Literature review questions and search terms utilised, per chapter

Chapter	Aim	Search Question	Search Terms	Exclusion Criteria
Chapter 3	1	What is the utilisation of HIV resistance testing after virological failure (VF) in European or high income settings and what factors are associated with having a resistance test performed?	("hiv infections"[MeSH Terms] OR "anti-hiv agents"[MeSH Terms] OR "hiv-1"[MeSH Terms]) AND ("drug resistance, multiple, viral"[MeSH Terms] OR "drug resistance, viral/genetics"[Mesh Terms] OR "mutation"[MeSH Terms] OR "genotype"[MeSH Terms] OR "drug resistance, viral"[Mesh Terms]) AND ("prevalence"[MeSH Terms] OR "epidemiology"[MeSH Terms] OR "prospective studies"[MeSH Terms]) NOT (transmitted[All Fields] OR transmission[All Fields])	Papers reporting on resistance testing from Asia, Sub-Saharan Africa or South America Case Reports Studies that focus on a single drug or a single mutation
	2	What is the probability of detecting drug resistance when performing a resistance test after VF and what are the risk factors for having drug resistance, given that a test was conducted?		
Chapter 4	1	What is the probability of VF among people receiving RAL from ART experienced?	("hiv infections"[MeSH Terms] OR "anti-hiv agents"[MeSH Terms] OR "hiv-1"[MeSH Terms]) AND "hiv integrase inhibitors"[MeSH Terms] OR ("raltegravir potassium"[MeSH Terms] OR ("raltegravir"[All Fields] AND "potassium"[All Fields]) OR "raltegravir potassium"[All Fields] OR "raltegravir"[All Fields]) AND (Viral Failure[All Fields] OR Virological Failure[All Fields] OR ("treatment failure"[MeSH Terms] OR ("treatment"[All Fields] AND "failure"[All Fields]) OR "treatment failure"[All Fields]) OR "Viral Rebound"[All Fields] OR "outcome"[All Fields] OR "outcomes"[All Fields])	Papers reporting on resistance testing from Asia, Sub-Saharan Africa or South America Case Reports Randomized controlled trials
	2	What is the prevalence of integrase resistance among people failing an INSTI?		
Chapter 6	1	How does resistance development depend on the level of viral replication?	("hiv infections"[MeSH Terms] OR "anti-hiv agents"[MeSH Terms] OR "hiv-1"[MeSH Terms]) AND ("drug resistance, multiple, viral"[MeSH Terms] OR "drug resistance, viral/genetics"[Mesh Terms] OR "mutation"[MeSH Terms] OR "genotype"[MeSH Terms] OR "drug resistance,	Paediatric studies Studies in specific sub populations (eg only women or TB co-infected patients)

			viral"[Mesh Terms]) AND ("prevalence"[MeSH Terms] OR "epidemiology"[MeSH Terms] OR "prospective studies"[MeSH Terms] OR “Rate” [All Fields] OR “Accumulation” [All Fields]) NOT (transmitted[All Fields] OR transmission[All Fields])	Studies that focus on a single drug or a single mutation Case Reports
Chapter 5	1	What is the effect of drug resistance on CD4 count changes among individuals maintained on a failing regimen after the start of ART?	("hiv infections"[MeSH Terms] OR "anti-hiv agents"[MeSH Terms] OR "hiv-1"[MeSH Terms]) AND ("drug resistance, multiple, viral"[MeSH Terms] OR "drug resistance, viral/genetics"[Mesh Terms] OR "mutation"[MeSH Terms] OR "genotype"[MeSH Terms] OR "drug resistance, viral"[Mesh Terms]) AND (CD4 Lymphocyte Count OR CD4-Positive T-Lymphocytes/drug effects OR CD4-Positive T-Lymphocytes/immunology AND (“transmitted” [All Fields] OR “naïve” [All Fields] OR “primary” [All Fields])	Paediatric studies Case Reports
Chapter 7	1	What is the effect of TDRM on CD4 count changes before the start of ART?	("hiv infections"[MeSH Terms] OR "anti-hiv agents"[MeSH Terms] OR "hiv-1"[MeSH Terms]) AND ("drug resistance, multiple, viral"[MeSH Terms] OR "drug resistance, viral/genetics"[Mesh Terms] OR "mutation"[MeSH Terms] OR "genotype"[MeSH Terms] OR "drug resistance, viral"[Mesh Terms]) AND (CD4 Lymphocyte Count OR CD4-Positive T-Lymphocytes/drug effects OR CD4-Positive T-Lymphocytes/immunology) AND (transmitted [All Fields] OR Naive [All Fields] OR Primary [All Fields])	Paediatric studies Case Reports

2	What is the effect of TDRM on viral load changes, including viral load set point, before the start of ART?	("hiv infections"[MeSH Terms] OR "anti-hiv agents"[MeSH Terms] OR "hiv-1"[MeSH Terms]) AND ("drug resistance, multiple, viral"[MeSH Terms] OR "drug resistance, viral/genetics"[Mesh Terms] OR "mutation"[MeSH Terms] OR "genotype"[MeSH Terms] OR "drug resistance, viral"[Mesh Terms]) AND (“Viral Load” [MeSH Terms] OR “Viral Replication” [All Fields] OR “RNA, Viral” [MeSH Terms]) AND (transmitted[All Fields] OR Naive[All Fields] OR Primary[All Fields])	Paediatric studies Case Reports

Conference abstracts were included provided that enough information to determine eligibility was available in the abstract. As the literature reviews were not the primary objective of this thesis, formal review protocols were not published, secondary reviewers were not used to screen the papers and meta-analyses were not conducted. Flow diagrams of studies screened, assessed for eligibility and included in the reviews are included in each chapter. The results are presented in a narrative format in order to provide a summary of the existing state of knowledge at the time the work was undertaken.

2.2. Data Sources

The data used in this thesis comes primarily from the EuroSIDA cohort, a large, prospective cohort study of HIV-infected individuals in Europe, Israel and Argentina. However, data from other HIV cohorts and clinics have also been used. In chapter 6, the UK Collaborative HIV cohort (UK CHIC) Study and the UK HIV Drug Resistance Database (UK HDRD) contributed data. Data from the ViroLAB collaboration, EU-Resist collaboration, London St Mary's and Royal Free Hospitals, and the University of Bari were used for chapter 7. These contributing clinics are referred to as the "EU-TDR" collaboration throughout the thesis. For each collaborative analysis, data was merged with the EuroSIDA study. More detailed descriptions of the design of the different studies can be found below. As the EuroSIDA cohort contributed the majority of the data to this thesis, the focus of this chapter is on the methodology and design of EuroSIDA.

Table 2.2. Chapters and data sources in this thesis

Chapter	Data Source	Data included until	Considered for Inclusion	Included
Chapter 3	EuroSIDA	<03/2013	18,473	8,469
Chapter 4	EuroSIDA	<02/2016	21,879	2,447
Chapter 5	EuroSIDA	<03/2015	18,914	
	UK CHIC/UK HDRD	<2014	47,201	5,357
Chapter 5	EuroSIDA	<02/2016	21,879	464
Chapter 7	EU TDR Collaboration	<2006	5,203	
	EuroSIDA	<03/2015	18,914	6,180

2.2.1. EuroSIDA: Study overview and coordination

EuroSIDA is a prospective observational cohort study that has been collecting data since May 1994 (425). To date, 21,880 patients from 108 centres in 35 European countries (Austria, Belarus, Belgium, Bosnia-Herzegovina, Bulgaria, Croatia, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, The Netherlands, Norway, Poland, Portugal, Romania, Russia, Serbia, Slovakia, Slovenia, Spain, Sweden, Switzerland, Ukraine, United Kingdom), Israel and Argentina have been enrolled (Figure 2.2). These countries are commonly grouped into different geographical regions, as can be seen in Table 2.3 below.

Figure 2.2. Countries and regions in EuroSIDA

Table 2.3. Countries included in each geographical region of Europe and Argentina

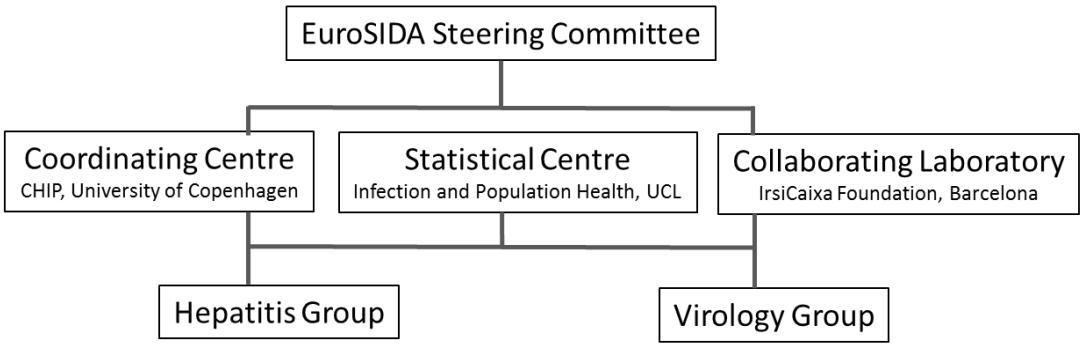
Southern	Central Western	Northern	Central Eastern	Eastern	Argentina
Spain	France	United Kingdom	Poland	Estonia	Argentina
Portugal	Belgium	Ireland	Czech Republic	Latvia	
Italy	Luxembourg	the Netherlands	Slovakia	Lithuania	
Greece	Switzerland	Denmark	Hungary	Belarus	
Israel	Austria	Sweden	Romania	Ukraine	
	Germany	Norway	Serbia	Russia	
		Finland	Bulgaria		
			Croatia		

The EuroSIDA study developed out of the AIDS in Europe retrospective cohort study initiated by Professor Jens Lundgren (University of Copenhagen) (426). The initial focus of the EuroSIDA

study was to report on prognostic factors and regional differences in HIV-related mortality and morbidity (427). Following the improvements in HIV treatment that dramatically prolonged survival for people living with HIV (PLWH), the focus of the EuroSIDA cohort has expanded. Although the study still aims to report on long term clinical, virological and immunological outcomes; analyses of clinically important co-morbidities, including Hepatitis B and C co-infection, renal function and cardiovascular disease have taken on greater importance (428). The latest round of data collection will exclusively enrol patients co-infected with Hepatitis C in order to provide data on the uptake and consequence of the roll-out of direct acting antivirals (DAA) for the treatment of Hepatitis C.

The study organisation is outlined in Figure 2.3. The management and coordination of EuroSIDA is done by the coordinating centre at the Centre for Infectious Disease Research (CHIP, Previously Copenhagen HIV Programme) at Rigshospitalet, University of Copenhagen. The coordinating team is responsible for questionnaire design, data collection, data entry and quality assurance. The EuroSIDA coordinating centre works in close collaboration with the statistical centre based in the Department of Infection and Population Health at UCL. The statistical centre provides statistical and analytical support to the study, but also leads a number of analyses and projects utilising data from the study with support from clinicians in the coordinating centre. Virological work, including genotyping for HIV and HCV, was done in London at the International Clinical Virology Centre until 2004. Since then this work has been undertaken at the collaborating laboratory at the IrsiCaixa Foundation in Badalona, Spain under the leadership of Dr Roger Paredes.

Figure 2.3. Organisation of the EuroSIDA study



The study is controlled by a steering committee consisting of 15 investigators as well as representatives from the coordinating centre, statistical centre and the collaborating laboratory. The steering committee is responsible for securing study funding and for approving research proposals and output from the study. Members of the steering committee are elected for a 5-year period. In turn these members elect a committee chair and a vice-chair from amongst themselves. The group communicates via phone conferences every two months and annually at one of the HIV scientific conferences. The EuroSIDA study group meets annually at an investigator face-to-face meeting, and a full list of the group can be found in Appendix I.

All research proposals submitted to EuroSIDA, including those from researchers outside the immediate study group, undergo a rigorous review process (429). All proposals must be submitted according to a standardised format. These are then sent for review by two independent members of the steering committee. Their responses are discussed at the subsequent EuroSIDA steering committee teleconference (TC), and any requested changes or additions to the proposals must be addressed before it is granted approval to proceed. The steering committee also review conference abstracts, presentations and journal articles before these are submitted for publication.

2.2.1.1. Inclusion criteria and recruitment

Individuals recruited into EuroSIDA are required to be HIV-positive, aged above 16, and are recruited in rounds (referred to as cohorts). Since 1994, there have been 9 cohorts, recruiting an average of around 2000 individuals each (Table 2.4). Initially, patients were enrolled only when their CD4 counts were below 500 (425), but this requirement was lifted in 1999. The inclusion criteria for cohort X have changed to reflect the evolving aims of EuroSIDA, and individuals are now required to be Hepatitis C antibody positive, as well as HIV positive, in order to be enrolled.

Table 2.4. Number of patients recruited per cohort in EuroSIDA

Cohort	Year	N
Cohort I	Spring 1994	3115
Cohort II	Winter 1995	1364
Cohort III	Spring 1997	2837
Cohort IV	Spring 1999	1225
Cohort V	Winter 2001	1223
Cohort VI	Winter 2003	2118
Cohort VII	Winter 2005	2458
Cohort VIII	Summer 2008	2254
Cohort IX	Spring 2012	2500
Cohort X	Status as of May 2016	3878 (80% of target recruitment)

After a centre agrees to participate in the EuroSIDA study, individuals are recruited consecutively until a pre-specified cap, set by the coordinating centre in collaboration with the participating clinic, is reached. Patients provide informed consent in accordance with local clinical guidelines; signed consent forms are held by the coordinating centre in Copenhagen. Clinicians are offered reimbursement for recruiting a patient, for each consequently submitted FU form and for submitting data relating to particular areas of interest. The reimbursement levels that were in place during the time-frame of this PhD thesis can be seen in Table 2.5.

Table 2.5. EuroSIDA reimbursement levels

Form/module	Current reimbursement (2015)	Future reimbursement (2017)
Follow-up form	13.60 €	20 €
HCV treatment form	50.00 €	30 €
HCV follow-up form	NEW	10 €
HCV adverse event form	50.00 €	50 €
HSR/liver toxicity form	161.64 $	50 €
HSR/liver toxicity form	-	-
CoDe[1]	161.64 $	20 €
Enrolment form	18.14 €	18.68 €
Plasma samples	5.67 €	5.84 €
Resistance data	5.67 €	N/A
D:A:D event form	161.64 $	N/A

1. *Causes of Death form*

2.2.1.2. Data items, collection and quality assurance

A number of clinical and demographic variables are collected for all EuroSIDA participants on a standardised Case Report Form (CRF) every 6 months. An overview of the items collected can be seen in Table 2.6 on page 83. It includes information on gender, age, ethnicity, mode of HIV transmission, CD4 and RNA data, stop and start dates for ART as well as data on AIDS defining illnesses (according to the 1993 CDC definition) and non-AIDS events. The EuroSIDA network of clinics has also been used to administer one-off surveys on the clinical management of HIV patients in Europe. Examples of such surveys include the Atripla Survey (430), which collected data on the use of Atripla® (efavirenz/emtricitabine/tenofovir) at different clinics in Europe, and the EuroSIDA clinic survey (431), which collected additional information on the clinical management of HIV-positive patients. Data collection is now done through an online system called RED-CAP (432), but initially physical copies of the forms were submitted to the coordinating centre where the data was entered into the EuroSIDA database manually.

A number of measures are taken by the coordinating centre to ensure that the quality of the data collected is of a high standard (428,433). This is done partly through annual monitoring of a number of sites in order to ensure accurate enrolment and data collection. Case notes are checked against the existing information submitted to the study for all clinical events and for a

random sample of 10% of patients. When a site is found to have incorrectly recorded data, feedback is provided to the centre and more frequent monitoring occurs to ensure that accuracy improves. There are also a number of data quality checks done by the data management team at the coordinating centre; these are outlined in detail in the quality assurance document published on the CHIP webpage (429) . Recently, the monitoring has been shifted from site monitoring to central monitoring at the coordinating centre.

Loss to follow-up (LTFU) is an important source of potential bias in many observational studies for two main reasons. Firstly, as patients with more chaotic lifestyles are more likely to be LTFU this has the potential to introduce systematic differences in the amount of FU time and information provided by individuals from different risk groups and demographics. Secondly, if many patients are LTFU it reduces the power of the study. Defining LTFU is challenging, especially for long-term conditions such as HIV, where patients may not be seen in clinic that often. A one-year gap in clinical data does not necessarily indicate that a patient is LTFU. A discussion around the challenges in defining LTFU and estimates of LTFU in EuroSIDA has been previously published (433). Measures are taken to help sites minimize the number of patients that are LTFU (433). Firstly, investigators are educated on the importance of continued follow-up and reporting. If a patient is LTFU, sites are contacted in order to see if more information can be obtained. And finally, sites with a high proportion of patients LTFU are approached to see if the underlying reasons for the high LTFU can be elucidated. These sites also receive extra support and training that can help reduce LTFU (433).

Table 2.6. Summary of data collected for the EuroSIDA study

Demographics	Gender	**Comments**
	Date of Birth	
	Mode of Infection	
	Country of Origin	
	Race	
	Weight	
	Height	
HIV: Laboratory Measures	RNA	
	CD4	
HIV: Virology	Date of resistance test	
	Result from resistance test	
HIV: Treatment	ART start and stop dates	
	Reasons for discontinuation	
	Adherence rating as reported by the treating physician	Not validated
HIV: AIDS	Date and type of opportunistic infections	
	Treatment against opportunistic infections	
	Date and Diagnosis of AIDS defining	
Death	Death date	
	Autopsy	

	CoDE case report	
Laboratory values	Serum total cholesterol	
	Serum HDL cholesterol	
	Serum triglycerides	
	S-creatinine	
	Haemoglobin	
	Platelet count	
	ALT	
	AST	
	INR	
	Bilirubin	
	S-lactate	
	S-amylase	
	Toxoplasmosis antibody	
	CMV antibody	
	Proteinuria	
Hepatitis	Hepatitis B antigen	Since 1997
	Hepatitis B antibody	
	HBV-DNA	
	Hepatitis C antibody	
	HCV-RNA	
	HCV-genotype	
	Copies of liver biopsy results	Since 2010
Clinical (non-AIDS)	Cardiovascular disease	
	Metabolic events	
	Other organ events	
	Treatment for clinical/non-AIDS conditions	
	Date and diagnosis of non-AIDS malignancies	
Other data	Blood Pressure	
	Smoking status	
	Family history of MI	
	Alcohol abuse (past or current)	Not validated
	Pregnancy outcome (if pregnant)	

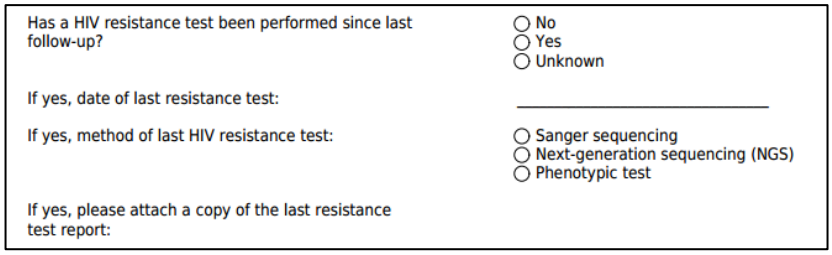
2.2.1.3. The sample repository

Since 1997, EuroSIDA has been collecting plasma samples for a plasma repository held at the coordinating centre in Copenhagen. Plasma samples of one ml are requested for all participants every six months, and these are shipped to the coordinating centre where they are frozen and stored in the plasma repository. The samples have been used for a variety of different projects relating to antiretroviral resistance, hepatitis, pharmacokinetics and biomarkers.

2.2.1.4. EuroSIDA resistance data

Data on drug resistance and resistance testing has been collected since 1997 in three different ways. Firstly, there is a question in RED-CAP where the clinician can indicate whether or not a person has been genotyped since their last clinic visit (Figure 2.4)

Figure 2.4. Screen shot of resistance collection for EuroSIDA (RED-CAP)



Secondly, a clinician can submit a report of genotypic data which is consequently added to a resistance database. The form of this report varies: occasionally a list of mutations is attached as part of a virological report, and in other instances FASTA files with sequences are attached electronically. The data is formatted by the virology team in Badalona and kept in a centralised database. For the most recent analyses included in this thesis, data on 5,196 resistance tests from 3,191 individuals submitted as paper forms were available. As the genotyping of these samples is done locally, the method used for genotyping and interpretation varies depending on the clinical standards and available technologies in each centre. Finally, genotypic data has also been added to the resistance database as a result of retrospective genotyping of plasma samples from the plasma repository. This genotyping is done by the collaborating laboratory for specific projects. In D40, data on 5,966 centrally performed resistance tests from 3,060 patients were available. Since many rounds of retrospective genotyping have been done over time, several different methods for genotyping have been used. The method used for genotyping also varies according to the project aim. In recent years there has been a shift towards using next generation sequencing methods for projects. This is the approach that was taken in Chapter 4, where the Illumina MiSeq platform was used to genotype people who were failing on an integrase inhibitor containing regimen. In total, D40 held genotypic data on 11,162 resistance tests from 5,265 patients.

EuroSIDA also collects a measure of adherence which is of considerable relevance to analyses of resistance data. However, this adherence measure is provided by the treating physician and is likely to at least partly be influenced by the results from VL tests. It has not been validated, has a high proportion of missing data and it is not clear to what extent this measure accurately

reflects patient adherence. Following the recommendation of the study PIs and SC, the adherence data has therefore not been used in this thesis.

2.2.1.5. Funding and ethics

At the time of writing (October 2015), primary funding for EuroSIDA was provided by the European Union's Seventh Framework Programme for research; technological development and demonstration under EuroCOORD grant agreement n˚ 260694. Support also included unrestricted grants by Bristol-Myers Squibb, Janssen R&D, Merck and Co. Inc., Pfizer Inc. and GlaxoSmithKline LLC. The participation of centres from Switzerland was supported by the Swiss National Science Foundation (Grant 108787). The study is also supported by a grant [grant number DNRF126] from the Danish National Research Foundation.

Each centre that contributes data to the EuroSIDA study has sought and been granted ethics approval from the relevant local and national authorities. A copy of each approval form is held at the coordinating centre in Copenhagen, and these are available per request from eurosida.rigshospitalet@regionh.dk**.** Where the local and/or national ethics committees require individual patient consent, this is taken before individuals can be enrolled in the study.

2.2.1.6. Summary of the EuroSIDA study to date

By February 2016, EuroSIDA had recruited 21,880 patients; a brief description of the characteristics of these patients at enrolment can be seen in Table 2.7. In the analyses done for this thesis, different inclusion criteria were applied and the numbers and characteristics of the study population may therefore vary from chapter to chapter. However, the overall baseline characteristics are presented here to give an indication of the study characteristics. Additional information on baseline characteristics is also presented per chapter.

Table 2.7. Characteristics of individuals in each study at enrolment[1]

		EuroSIDA (D42, 2016)		UK CHIC (2014)		EU-TDR (2010)	
		N	%	N	%	N	%
Total		21,880	100	47,201	100	5,203	100
Gender	Male	15,931	72.8	34,202	72.4	3,721	71.5
	Female	5,573	25.5	12,995	27.5	1,179	22.7
	Missing	376	1.7	4	0.01	303	5.8
Enrolment Age	Median [IQR], Years	37.5	31.3-45.6	37.7	32.1-41.7	35.7	29.5-42.5
	Missing Age	402	1.8	4	0.01	60	1.1
Ethnicity	White	18,959	86.7	24,612	52.1	*Not available*	-
	Non-white	2,397	11.0	19,690	41.7	-	-
	Missing	524	2.4	2,899	6.1	-	-
Risk Group	MSM	7928	36.2	23,341	49.5	2,099	40.3
	IDU	6073	27.8	1,694	3.6	462	8.9
	Heterosexual	6020	27.5	17,191	36.4	1,663	32.0
	Other	1356	6.2	1,862	3.9	654	12.6
	Missing	503	2.3	3,113	6.60	325	6.3
Region	Southern	5,657	25.9	0	0	*Not available*	-
	Central Western	4,886	22.3	0	0	-	-
	Northern	4,964	22.7	47,201	100	-	-
	Central Eastern	2,532	11.6	0	0	-	-
	Eastern	3,199	14.6	0	0	-	-
	Argentina	642	2.9	0	0	-	-
Enrolment CD4 count	Median [IQR], Cells/mm^3	340	180-520	315	150-496	434 (298-599)	

	Missing enrolment CD4	534	2.4	26,910	57.0	0	0
Enrolment Viral Load	Median [IQR], copies/ml	200	39-9,505	16,188.5	553-99,900	29,500	8,243-103,140
	Missing enrolment VL	6,092	27.8	31,511	66.8	606	11.6
Hepatitis C	Yes	6,511	42.0	748	1.2	*Not available*	-
	No	9,003	58.03	8955	19.0	-	-
	Missing	6,366	29.1	37,498	79.4	-	-
Hepatitis B	Yes	1,018	6.7	655	1.4	*Not available*	-
	No	14,182	93.3	10,870	23.0	-	-
	Missing	6,680	30.5	35,676	75.6	-	-

1. Approximated by the first available CD4 count for the EU-TDR clinic data, as a first visit date was not available.

2.2.2. UK CHIC: study overview and coordination

The UK CHIC study collates routinely collected data from a number of HIV and Sexual Health clinics from across the UK. It was set up in 2001 by Professor Caroline Sabin (UCL), and includes all data on patients seen at participating centres since January 1996 (434). The overall aim of the study is to create a nationally representative database of individuals who receive care for HIV in the UK, in order to describe long-term outcomes of cART and factors associated with virological and immunological responses to cART. Currently, 21 centres contribute data to the study (Appendix II). The study complements the existing UK HIV surveillance data by collecting more detailed information than it is possible to do using current routine surveillance structures.

UK CHIC is coordinated by a team of epidemiologists and statisticians from the Department of Infection and Population Health, UCL. The steering committee has 31 members, and consists of representatives from the centres that contribute data, the Medical Research Council Clinical Trials Unit (MRC-CTU), Public Health England (PHE) and community organisations (HIV i-Base). The steering committee meets every 3-6 months, and is responsible for approving research proposals and outputs from the study. Members of the steering committee are also encouraged to submit their own research proposals. There are also a number of research sub-groups that study areas of specific interest. These include renal disease, viral hepatitis, pregnancy and ageing (434).

2.2.2.1. Inclusion criteria and recruitment

Individuals included in the UK CHIC study are HIV-positive individuals aged 16 and above who have attended one of the collaborating centres for care at any time since 1/1/1996. Healthcare centres do not actively recruit patients, but instead submit copies of the data they already collect on all patients seen for care who meet the inclusion criteria.

2.2.2.2. Data collection and quality assurance

Data for UK CHIC is submitted to a database held at the MRC CTU electronically, and include demographics, laboratory measures including CD4 counts and viral load measures, data on ART history and AIDS diagnoses (435). The data is held in a secure, integrated, relational database, and entries are pseudo-anonymised. Updates of the dataset are provided by participating centres on an annual basis.

A number of procedures to ensure high accuracy and completeness of the data submitted are in place (Figure 2.5) (434). This involves a comparison of the data held in clinic databases with the clinical case notes for a random selection of 1% of patients' records at each centre. Patients who attend more than one clinic have their records linked on the bases of their

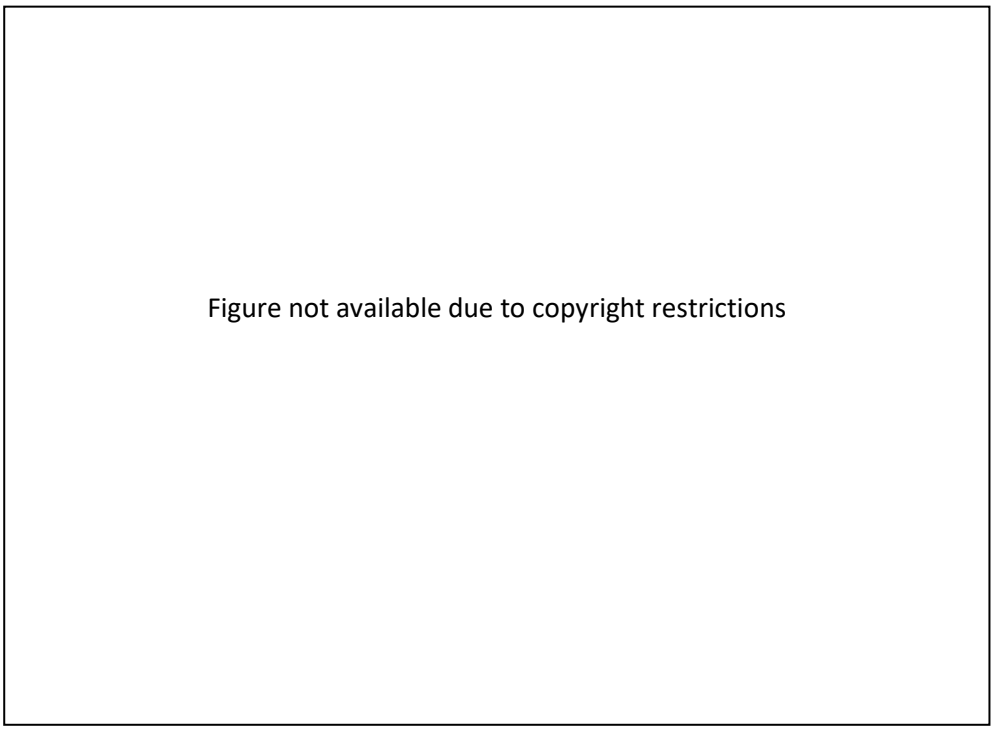
soundex code, date of birth and other clinical information. Details on the matching algorithm have been previously published (435).

Figure 2.5. Data collection procedure in UK CHIC

2.2.2.3. The UK HIV Drug Resistance Database (UK HDRD)

The UK HDRD is a repository for all the resistance tests performed as part of routine care since 2001 in the UK (436). By the end of 2013, the database held over 114,000 test results, primarily viral gene sequences. The database has a steering committee that meets regularly Appendix III), and is coordinated by the MRC CTU (Medical Research Council – Clinical Trials Unit) at UCL. Data is submitted to the HDRD on annual basis and imported into a central SQL database (Figure 2.6). The submitted nucleotide sequences are consequently processed through the Stanford University Genotyping Resistance Interpretation Algorithm (394). This produces data on amino acid mutations and drug susceptibility. The resistance database is annually linked with existing clinical cohorts, including UK CHIC, in order to provide data on resistance patterns for epidemiological analyses. As the HDRD collects data on all resistance tests done as part of clinical care, the UK is one of few European countries that can provide nationally representative data on HIV drug resistance without conducting prevalence surveys.

Figure 2.6. Organisation of the UK HIV Drug Resistance Database



2.2.2.4. Funding and ethics

The UK CHIC study and UK HDRD are been funded by the MRC, UK. In August 2014, the UK CHIC Study and the UK HIV Drug Resistance Database (UK HDRD) were jointly awarded a further 5 years of support from the MRC, enabling the projects to continue until August 2019 (grant code M004236).

UK CHIC has been approved by a multi-centre research ethics committee as well as by local ethics committees. As the data is pseudonomised, individual patient consent is not necessary for the study.

2.2.2.5. Summary of the UK CHIC/UK HDRD

The characteristics of the patients enrolled in UK CHIC (linked with the UK HDRD) at their first visit date can be seen in Table 2.7 (page 87).

2.2.3. European Transmitted Drug Resistance (EU TDR) Collaboration: study overview and coordination

Part of the data used in chapter 7 comes from a collaboration, which for simplicity, is referred to as the European Transmitted Drug Resistance collaboration (EU-TDR). This database was obtained by merging the databases of two European collaborative consortiums on antiretroviral drug resistance (the Virolab Consortium and the EuResist Consortium) with data from three additional UK centres and one Italian centre caring for HIV-positive patients (St. Mary's Hospital, Imperial College London; Royal Free Hospital and "Policlinico" hospital, University of Bari). The database created as part of the ViroLab project (http://www.virolab.org/about-virolab.html) contained resistance information as well as clinical and demographic data of patients who had a HIV genotypic resistance test as part of clinical practice in seven different European clinics (Tropical and Infectious Diseases clinic, University of Brescia (Italy); Catholic University of the Sacred Heart in Rome (Italy); Hospital Germans Trias i Pujol, Foundation IrsiCaixa of Barcelona (Spain); Rega Institute for Medical Research, Catholic University of Leuven (Belgium)). The EuResist integrated database (http://engine.euresist.org/database/) included clinical, demographic and sequence data collected in Italy (ARCA database), Germany (AREVIR database), Sweden (Karolinska Infectious Diseases and Clinical Virology Department), Luxembourg (Retrovirology Laboratory, CRP-Santé), Portugal (Instituto de Higiene e Medicina Tropical) and Russia (Ivanovsky Institute of Virology). In order to avoid duplicates, patients from Catholic University of the Sacred Hearth included in the ARCA database were excluded from the merge.

2.2.3.1. Data collection and inclusion criteria

Participating clinics were requested to send specific data items on patients meeting the inclusion criteria for the aims of the collaboration, which related to the study of transmitted drug resistance in relation to the viral load, CD4 counts and viral fitness estimates. The inclusion criteria for data submission were the existence of a resistance test performed between 1996-2009 while the patient was naïve to ART, being above the age of 16 and HIV-positive. The data items requested were gender, age, mode of infection, country of origin, CD4 and VL measurements taken before the start of ART, as well as the date and result of the resistance test and subtype identification if done. All data were sent to the University of Brescia, where an integrated database of the patient data was created. Primary data collection occurred once in 2007, followed by an update and expansion of the database to include the non-ViroLAB laboratories in 2009. This expanded database was used for the analysis in Chapter 7.

As the dataset is a collaboration, the data quality checks in place differ depending on the clinic. No centralised queries or data quality checks are in place, and the accuracy of the existing dataset reflects accuracy of the data submitted to the study.

2.2.3.2.Funding and ethics

The data collected for the EU-TDR collaboration was supported by the European Commission (FP6 Virolab Project Grant IST-027446; FP6 EuResist Project Grant IST-027173) and in part by a grant from the Fonds voor Wetenschappelijk Onderzoek Vlaanderen (FWO G.0692.14N).).
Ethics was granted by national and local committees to each of the collaborating clinics.

2.2.3.3. Summary of the EU-TDR dataset

A summary of the characteristics of the participants in the EU-TDR cohort at their first available date can be seen in Table 2.7 (page 87).

2.3. Statistical Methods

Several different statistical methods have been used throughout this thesis, and a summary of which methods were used for each chapter can be found in Table 2.8. For each chapter, descriptive statistics and basic univariable tests were used in addition to regression models. All methods, together with the steps taken before analysis, are described in general terms below and in more detail in each particular chapter.

Table 2.8. Key outcomes and statistical methods, per chapter

Chapter	End-point(s)	Main Statistical Methods
Chapter 3	(1) Proportion with a resistance test after VF (2) Proportion with detected drug resistance after a resistance test	Logistic Regression with generalised estimating equations (GEE)
Chapter 5	(1) Time to VF after receiving Raltegravir	Kaplan-Meier, Cox Regression
Chapter 6	(1) Change in CD4 count	Principal Component Analysis, Linear mixed models
Chapter 4	(1) Rate of resistance development	Poisson regression with GEE
Chapter 7	(1) Change in CD4 count (2) VL set-point	Principal Component Analysis, Linear mixed models

2.3.1. Data cleaning and management

In addition to the quality assurance undertaken at the coordinating centre, a certain amount of data management and cleaning is undertaken by the statistical centre depending on the requirements of each analysis. Such data management can include the selection of an appropriate study population according to pre-specified inclusion criteria and the preparation of time-updated variables. As EuroSIDA collects data from routine clinic appointments, data is not collected at specific dates or at a specific point beyond baseline. This means that measurements for an individual are not always taken at the same time-points. When doing analyses involving time-updated data, it is therefore often necessary to approximate the value of a particular variable on a particular date. Throughout this thesis, this has mostly been done by carrying forward the last recorded value until a new value occurred, regardless of the lag-time. Other strategies for dealing with missing data are discussed in Section 2.3.5.4. It is also common that the data is reformatted from wide (one row per individual) to long (multiple rows per individual) to suit the requirements of different analyses.

The data cleaning procedures undertaken vary according to the requirements of each analysis, but it commonly involves finding and cleaning out duplicate values, removing implausible dates or implausible amino-acid substitutions in the resistance data.

2.3.2. Descriptive statistics

Descriptions of categorical data were done using proportions, and for continuous variables the mean (normally distributed data) or median (skewed data). For continuous variables, the spread was quantified using the standard deviation (normally distributed data) or the interquartile range (skewed data). Where relevant, graphical representations of the data (such as histograms and scatterplots) were used to assess the spread and distribution of the data, as well as to identify potential outliers.

2.3.3. Significance tests

Following a descriptive investigation, simple statistical tests are often used to compare baseline characteristics and to explore the research question. Statistical tests generate a p-value; the probability of obtaining results at least as extreme as those observed if the null hypothesis (H0) is true (437). The smaller the p-value is, the greater the evidence against the H0, the hypothesis of a null/no effect/difference. By convention, a p-value less than 0.05 is often used as indicating whether or not a result is 'statistically significant', that is, small enough to provide reasonable evidence against the null hypothesis. Despite the fact that denoting results as significant or not based on this cut-off is very common, it has been widely criticised as it encourages a focus on an arbitrary cut-off point and discourages reporting of the actual p-value obtained (438–442). Throughout this thesis, results are interpreted using p-values in conjunction with effect sizes and confidence intervals, as well as the likely clinical importance and plausibility of the findings (439,443). Multiple testing can impact the probability of finding a low p–value, and the implications of this are discussed below in section 2.3.3.1.

The choice of statistical test depends on the type of data. In order to test for differences in a binary outcome between two or more levels of a categorical variable, Pearson's chi-squared test was used. For small sample sizes where the expected cell counts are estimated to be less than 5, the chi-squared test is not reliable and I instead used Fisher's exact test (437). For binary exposures, the t-test was used to test for differences between means for normally distributed continuous data and the Wilcoxon rank-sum test comparing medians for skewed data. For categorical exposures with more than two categories, ANOVA was used to test for differences in normally distributed continuous data and the Kruskal-Wallis test for skewed data (437).

2.3.3.1. Multiple testing

As previously mentioned the probability of finding a low p-value increases with the number of tests conducted (441). There are methods to correct for multiple testing, and these are appropriate in certain instances, notably where a very large number of tests are done in the absence of a-priori hypotheses regarding the expected associations. For most research questions however, methods that control for multiple testing can be overly conservative and it is instead recommended to base the interpretation of the results on confidence intervals and effect sizes in addition to the p-value, rather than correcting for multiple testing (444). In this thesis I corrected for multiple testing when I tested large (>30) numbers of exposures selected on the basis of arbitrary rules or data-driven approaches, such as expert lists of mutations and prevalence thresholds (chapter 5 and 7). In these instances I corrected p-values using the false discovery rate (FDR) as described by Benjamini and Hochberg (445). This involves sorting the p-values in ascending order and adjusting each p-value using their percentile rank (446). The resulting adjusted p-value is referred to as a q-value, and it represents the probability that a particular finding is a false positive. I used the SAS procedure *PROC multtest* to automatically calculate q-values.

2.3.4. Regression models

In epidemiology, regression models are commonly used to estimate the effect of one or more exposures (independent variables) on an outcome (dependent variable). In univariable models, the effect of one exposure variable on the outcome is estimated. Multivariable models, which contain more than one exposure variable, estimate the effect of an exposure on the outcome conditional on all the other variables in the model. The main benefit of using regression models over the statistical tests introduced in section 2.3.3 is that they can take the effect of more than one exposure into account, that is, control for the potentially confounding effect of one or more exposure variables on the association of interest (437). Confounding is introduced separately in section 2.3.5.1, and a brief introduction to the regression models used in this thesis is provided below.

2.3.4.1. Linear regression

Linear regression models are used to estimate the effect of one or more predictors ($x_1, x_2, \ldots, x_n$) on a continuous outcome (y). Linear regression models take the form described in Equation 2.1 below.

Equation 2.1. Linear regression

$$y = \beta_0 + \beta_1 x_1 + ..\beta_n x_n + \varepsilon$$

The β values represent the regression coefficients, or parameters, associated with the exposure variables. The estimated value for a given β represents the impact on *y* of a one-unit increase in *x* when all other variables are held constant. The β_0, or intercept, represents the value of *y* when all other explanatory variables are zero (437). Although linear regression was not applied directly in this thesis, I utilised linear mixed models in chapter 5 and 7. These models are extensions of the linear regression equation that can deal with repeated measures, and they are discussed in more detail in section 2.3.5.3

2.3.4.2. Logistic regression

When outcome variables are binary (for example, a yes or no outcome) rather than continuous, logistic regression models are used. Logistic regression models follow a similar form to linear regression, although the outcome variable is transformed using a link function, in this case the logit function (447). The resulting model describes the linear relationship between the logit(p) and one or more exposures as can be seen in Equation 2.2, where p is the probability of the outcome event occurring and logit(p) is the natural logarithm of the odds of the outcome.

Equation 2.2. Logistic regression

$$Logit(p) = \beta_0 + \beta_1 x_1 + ..\beta_n x_n + \varepsilon$$

Given that 1 represents an event occurring, the parameter estimates β will give the log odds of y=1 given a one unit increase in x when all other values are held constant. By taking the exponential of the β terms an odds ratio can be derived (447).

2.3.4.3. Survival analysis

Data where subjects are followed over periods of time until a pre-specified event occurs are called survival data (437). A key feature of survival data is that observations can be censored; which means that even if an individual does not experience the event of interest during their follow-up time, their follow-up time can be stopped ('censored') at a pre-specified time. Data can be either right-censored, meaning that the survival time ends at the last know visit, or left-censored, which means that follow-up available before a defined baseline is not taken into account (437). Analysis of survival data, called survival analysis, involves statistical methods that allow us to take into account the varying length of follow-up produced by the censoring of data.

Biomedical measures such as viral load values can also be censored, as the tools used to measure such variables often cannot quantify exact values below a certain limit of detection (LOD). This also results in censored data, as although the exact value of the variable is unknown, it is known that the measured quantity was below the LOD. In this context, the data is referred to as truncated. In this thesis a simple approach of replacing the value <LOD with LOD was taken.

2.3.4.4. Kaplan-Meier

The distribution of survival times can be described by a mathematical function called the survival, or survivor function (Equation 2.3).

Equation 2.3. Survival function

$$S(t) = 1 - F(t)$$

Here F(t) gives the probability that the event has occurred by duration t, and S(t) therefore the probability of the event not having occurred by duration t. It is often of interest to estimate what S(t), or the probability of survival to at least time t, is. We can do this using a method known as Kaplan Meier. The Kaplan Meier estimator calculates the probability of survival in given time-intervals within the whole period of follow-up, with subjects censored before the specific time-point excluded from the denominator. The cumulative probability of survival from baseline until time t is then calculated by multiplying the probability of survival at each time interval. This is shown in Equation 2.4, where n_i is the number of individuals still at risk at the beginning of time interval t_i, and di the number of events during time t_i.

Equation 2.4. Kaplan Meier estimator

$$\hat{S}(t) = \prod_{t_i<t} \frac{n_i - d_i}{n_i}$$

The resulting estimates can be plotted as a Kaplan Meier, or survival, curve. These graphs are characterised by a series of horizontal steps and 'drops' which represent the occurrence of an event. The difference between two survival curves is most commonly tested using the log-rank test (437).

2.3.4.5. Cox proportional hazards regression

In order to estimate the effect that a number of exposures have on survival, Cox Proportional Hazards Regression can be used. This allows the natural logarithm of the hazard to be modelled as a linear function of a number of exposure variables (Equation 2.5).

Equation 2.5. Cox regression

$$Log(h(t)) = log(h_0(t)) + \beta_1 x_1 + .. \beta_n x_n$$

The hazard is defined as the instantaneous probability of having an event at time t, conditional on the event not having occurred by that time, and it is mathematically related to the survival function. Cox regression assumes that the relative hazards do not change over time ("proportional hazards"). Whether this assumption holds can be investigated in many different ways. In this thesis the proportional hazard assumption was assessed by testing for an interaction between given exposures and time (437). Using Cox regression allowed me to derive hazard ratios and 95% CI.

2.3.4.6. Poisson regression

Another model that can be used to analyse data with varying follow-up time is Poisson regression. Poisson takes a form similar to linear regression, although the outcome in this case is a count variable. Poisson regression uses a link function to transform the mean response Y to the natural logarithm of Y (Equation 2.6) (447).

Equation 2.6. Poisson regression

$$Log(y) = \beta_0 + \beta_1 x_1 + .. \beta_n x_n + \varepsilon$$

In order to use Poisson regression to model a rate rather than counts, we need to use the number of events per exposure time (Y/exposure time, i.e. the rate) as the outcome rather than just the count (Y). By multiplying both sides of the regression equation with the exposure time (t), the exposure time variable can be moved to the right-hand side of the equation. As both sides of the equations are log-link transformed, the final regression equation will have the natural logarithm of the exposure time added to the regression coefficients (Equation 2.7).

Equation 2.7. Poisson regression with an offset

$$Log(y) = \beta_0 + \beta_1 x_1 + .. \beta_n x_n + log(t) + \varepsilon$$

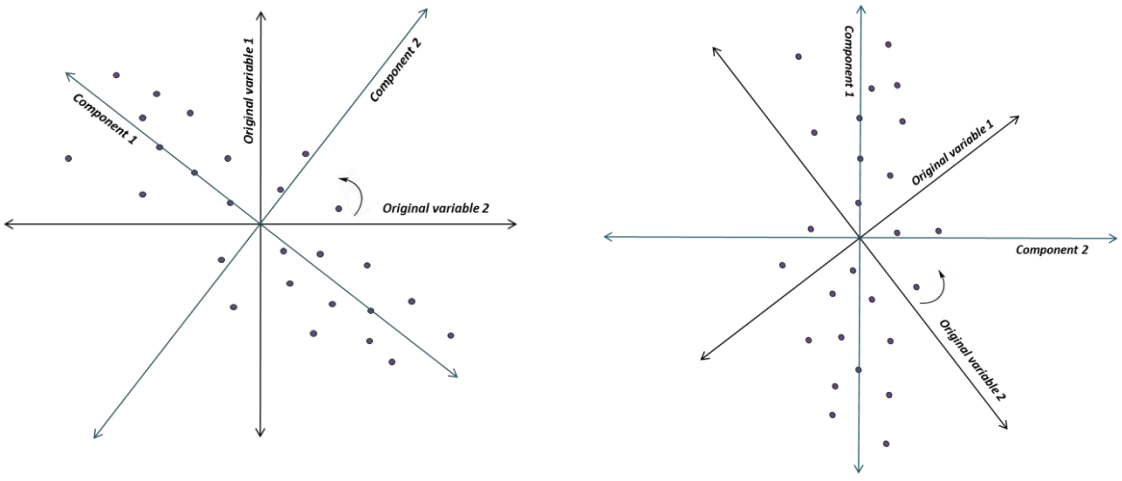
The natural logarithm of the exposure time in the above equation is called an offset variable, and its regression coefficient is constrained to be 1. After including the offset, the exponentiated outcome variable represents the incidence rate (448). Poisson regression can therefore be used to derive incidence rate ratios, together with 95% CI and p-values. In a model with only the intercept β_0, this term will equal the incidence rate in the population. Cox and Poisson regression can both be used for time to event data, although there are some key

differences between these two modelling approaches. In Poisson regression, the baseline rate is modelled directly and assumed to be constant over time within strata of the included exposures (437). In contrast, the baseline hazard is not directly modelled in Cox regression, and therefore no assumptions regarding its shape have to be made. This makes Cox regression a semi-parametric model. The hazard can also vary over time without breaking any model assumptions, presuming that the ratio of the hazards remains constant. This means that for data where the rate of the outcome is expected to change quickly, Cox regression may be more appropriate than Poisson regression. However, in most instances changing rates can be taken into account by including time-updated covariates in a Poisson model, and both types of models tend to give similar answers (437).

2.3.5. Principal Component Analysis

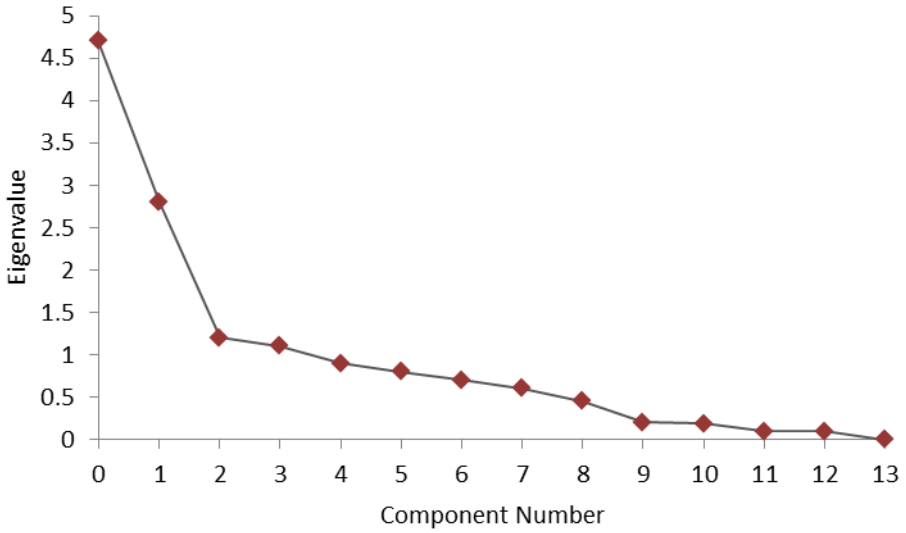
Principal component analysis (PCA) is a variable reduction technique that takes a number of correlated variables and transforms them into a smaller set of variables that are no longer linearly correlated (449). These new variables are referred to as principal components. Although a detailed explanation of the mathematics underlying PCA is outside the scope of this thesis, it can be understood graphically as a procedure which rotates values in a dataset into a set of new positions (450). These positions are chosen so that the axes of the new dataset lie in the direction of the greatest variability (Figure 2.7). The new axes are the principal components, and the transformed data values represent values on a given principal component. It is common to choose new axes, or components, that lie perpendicular to each other, as this ensures that the components are linearly unrelated. This type of rotation is called an orthogonal rotation, and a commonly used orthogonal rotation is the Varimax rotation (451).

Figure 2.7. A basic illustration of the PCA principle



The number of principal components extracted from a PCA is always the same as the number of variables entered into it, but the amount of the total variance that is explained by each component differs. What proportion of the variance that is explained by a given component is given by its eigenvalue (450). As the aim of PCA is to reduce the number of variables in a dataset, a decision has to be made as to how many components should be retained. A common strategy is to retain all components with an eigenvalue greater than one, which indicates that a component explains more of the total variance than a single variable (451). Another way is to utilise a scree plot. A scree plot is a plot of the components against their eigenvalues. The number of components to retain is indicated by a breakpoint (Figure 2.8), and it is common practice to retain all components above a certain breakpoint (451). In this thesis the latter strategy has been chosen, as retaining all components with eigenvalues larger than one can result in a very large number of components if the dataset is large and the variables entered into the PCA only weakly correlated.

Figure 2.8. Example of a scree plot



As described above, each variable entered into the PCA will have a certain value on each extracted component. If a variable has a high value on an extracted component this indicates that it contributes strongly to that component, and it is said to "load" onto that component. A cut-off of 0.4 is commonly used to identify significant loadings (451). By looking at which variables load onto a component, it is possible to construct interpretations of what pattern a given component is describing. I used PCA as a tool to study patterns of mutations. Mutations were entered as binary variables into the PCA, and clusters of mutations were identified by studying which mutations loaded significantly onto each component.

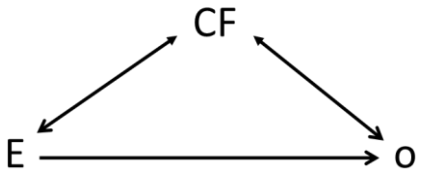
It is possible to use the loadings of variables to construct component based scores for each individual in the dataset. These scores indicate how closely an individual's data pattern aligns with that described by the extracted components. I used component based scores to study the impact of clusters of mutations on pre-specified outcomes (see Chapter 5 and 7). For simplicity, a binary cut-off point using the 75th percentile was used to categorise an individual's mutational pattern as either "mostly conforming" or "not conforming" to a mutational pattern.

2.3.6. Issues in statistical modelling

2.3.6.1. Confounding and effect modification

A confounding factor (CF) has classically been defined as a variable that is associated with the exposure (E), is an independent risk factor of the outcome (O) and not on the causal pathway between the exposure and outcome (Figure 2.9).

Figure 2.9. Classical illustration of confounding



Source: (452)

In this representation, the confounding factor can be seen as a common cause of both the outcome and the exposure. The presence of confounding can both decrease and amplify the observed effect of an exposure on an outcome, and in some instances even reverse the observed association. A typical confounder in HIV research and indeed most epidemiological studies is age. For example, when studying the effect of a given treatment on mortality, age can act as a confounder as it determines the probability of death and it is conceivable that the probability of receiving a particular treatment will vary with age. In this hypothetical example age is a common cause of both receiving the treatment and death, and it is not on the causal pathway.

It is important to account for confounding, either through study design or analysis. Accounting for confounding through study design can be achieved through randomization (if the study size is sufficiently large), through restricting entrance into the study to individuals who fall into a specific category of the confounder or through matching, where subjects are selected so that levels of the confounder are distributed equally in all study groups (447). Confounding can also

be adjusted for in multivariable models. By including a suspected confounder as a covariate in a regression model, inferences for the exposure of interest are done conditional on the confounding variables. The benefit of controlling for confounding in statistical models is that many confounders can be taken into account simultaneously (453).

A concept similar to but distinct from confounding is that of effect modification. Effect modification refers to the process by which an exposure has a different effect on the outcome in different strata of an effect modifier. Effect modification is sometimes called interaction. Interactions can be quantitative (same direction of effect, different magnitude) or qualitative (opposite direction of the effect). The first is sometimes called synergy, the latter antagonism. For a hypothetical example of effect modification, consider the effect of a drug on the risk of death. If a particular drug decreases the risk of death among men whereas it increases the risk of death among women, this would be an example of qualitative effect modification by gender.

Interactions may be of clinical or policy-related interest, and the aim should be to describe rather than to adjust for them. It is not routine to test for all possible interactions, as statistical significance is dependent on the sample size (452) and the likelihood of finding a false positive increases with the number of tests done (see Section 2.3.6.5). Instead, possible interactions that are deemed to be of clinical interest (and therefore, which should be investigated) are decided upon a-priori.

2.3.6.2. Directed acyclic graphs and model selection

In this thesis, I used the findings from my literature reviews and discussions with clinical and statistical experts to identify potential confounders or relevant risk factors. I also used Directed Acyclic Graphs (DAG) as a visual aid to support the selection of variables for inclusion in the multivariable models where the aim was to identify the casual effect of a single exposure on an outcome (454–457). DAGs are graphical representations of assumed relationships between the exposure of interest, covariates and the outcome. DAGs aim to identify a set of minimally sufficient confounders that need to be adjusted for in order to estimate a causal effect, by taking into account the potential for biases that can be induced through adjustment (458–461). A key to the symbols commonly used in a DAG (as drawn by the program DAGitty) can be found in Figure 2.10. below, and an example DAG shown in Figure 2.11.

Figure 2.10. Key to symbols used in DAGs

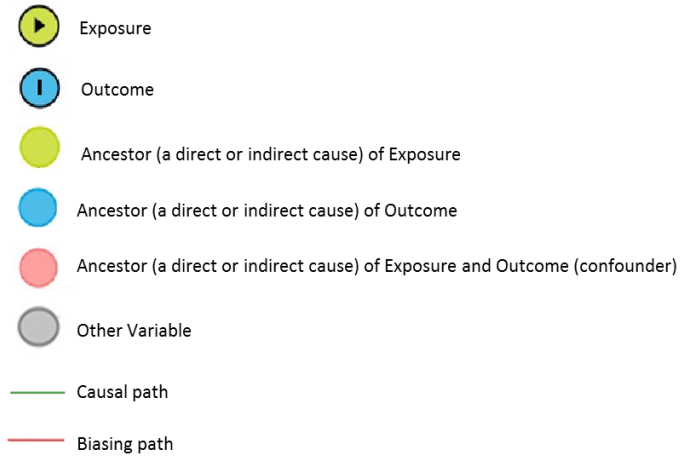
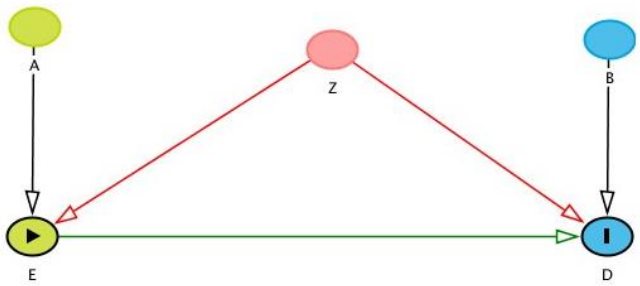


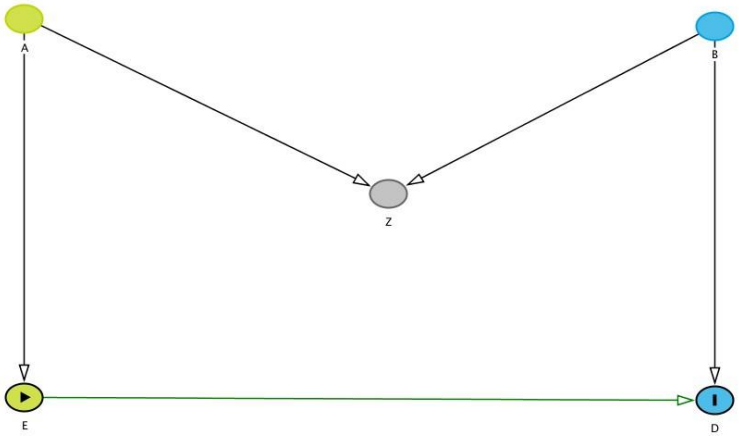
Figure 2.11. A simple example DAG



In the DAG above, the exposure (E) is associated with an outcome (D). In this example, there is one confounder Z, which is a common cause of both E and D. This is the classical definition of confounding. A is a cause of the exposure, but as it is not a cause of the outcome, it is not a confounder. B is associated with the outcome, but as it's not a cause of the exposure, it is not a confounder. This DAG implies that only adjustment for Z is necessary to estimate the direct total effect of E on D, and that additional adjustment for A or B would be unnecessary.

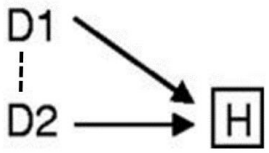
DAGs have been used in epidemiology to illustrate the types of biases that can be introduced through adjustment from confounders (458,461–463). One such potential bias is called collider-stratification bias (458,459). Briefly, a collider is any variable that is the descendant (or effect) of any two variables. An illustration of a collider (Z) is provided in Figure 2.12 below.

Figure 2.12. Illustration of a collider



In Figure 2.12, Z is a collider as it is a descendant of two different variables: A and B. Bias arises when such a collider variable is adjusted for, as associations among the causes of a collider change upon adjustment for it. Using the illustration provided in Figure 2.12: if Z is adjusted for, this will induce an association between A and B that was not previously there, which can introduce bias if the aim is to accurately describe the association between E and D. An applied example of collider-stratification bias is a classical epidemiological bias, Berkson's bias (464,465). Berkson's bias can occur when both cases and controls in a case-control study are sampled from a hospital. If the aim is to describe an association between two diseases, sampling participants from a hospital could make it appear as if two diseases are associated even though they are not, because people with two diseases are more likely to be hospitalised than people with one disease. In other words, selecting people conditional on their hospitalisation (H), will introduce a spurious association between disease one (D1) and disease two (D2). This is illustrated using a DAG in Figure 2.13.

Figure 2.13. Berkson's bias, illustrated using DAG's (adapted from Snoep et al (464))



Graph theory generalises an intuitive situation such as Berkson's bias, and predicts that bias can be introduced following stratification on any collider in given scenarios (458). This illustrates why, when the aim is to estimate a causal effect of one exposure on one outcome,

DAGs can offer some advantages compared to traditional epidemiological methods, such as forward/backward selection or change-in-estimate approaches in terms of guiding the model building. However, DAGs rely on a set of assumptions. The key assumption is that the DAG accurately reflects the true causal relations between the variables, that no variable that causes any pair of variables is missing and that variables are measured without error. Although these are strong assumptions, all model building strategies imply a particular causal structure (466). A benefit of DAGs is that they make the assumptions regarding the causal relationships transparent, which allows it to be critically assessed and improved (457).

I used the free open source software DAGitty (467) to construct DAGs for the main models in Chapters 5, 6 and 7, where the aims were to estimate causal effects. For these chapters, DAGs were used after potential confounders had been identified through literature reviews and discussions with experts in the field. Their function was as a visual aid, representing the thought process behind covariate adjustment, and as a check to ensure that bias, such as collider-stratification bias, was not introduced through over-adjustments.

2.3.6.3. Dealing with repeated measures

Including repeated measures taken over time on the same individual introduces correlation into a dataset. This is because measures taken from the same individual are likely to be more similar than measures taken from different individuals. This can overinflate the power of an analysis unless it is taken into account. To deal with repeated measures (Chapter 3, 5, 6 and 7) I used different techniques. In Chapter 3 and 6, generalised estimating equations (GEE) which account for the correlation through the specification of a 'working' correlation matrix were used (468). These allowed me to estimate population-averaged odds or rate ratios for the association between the risk factors and the outcomes. For Chapter 5 and 7 I used mixed models, which include random as well as fixed effects (469). Specifying a variable as a random effect allows predicted values for that variable to vary across individuals. Fixed effects on the other hand are constrained to have the same predicted coefficients across subjects. Mixed models combine subject-specific estimates from random effects to derive an overall regression parameter that is corrected for the correlation in the data. The key difference between GEE and mixed methods approaches is that the parameter estimates derived from mixed models are subject-specific, while those estimated in GEE are population-averaged. Although these differences are rather minor, it does influence the interpretation of the effect estimates. However, for the linear mixed model applied in this thesis, the parameter estimates are equal to the population-averaged estimates (470). The other main difference between GEE and

mixed models lie in that mixed models estimate and model the variance and covariance explicitly, whereas the covariance is treated as a nuisance parameter in GEE.

2.3.6.4. Dealing with missing data

Missing data is a common problem in analyses of cohort studies. In this thesis I used two different approaches in situations where the value of a covariate was missing for some of the participants. For variables where the amount of missing data was low and therefore did not drastically reduce the analysis population, I used a complete-case strategy whereby data lines with missing data was excluded from the model. This method can produce unbiased estimates when the data is missing completely at random, although this is rarely the case (447). However, when the number of individuals with missing data was large, an indicator variable for the missing category was included in the analyses. This increases statistical power and reduces the risk of selection bias, but may also introduce bias (447). Although advanced methods to account for missing data, including multiple imputation, may be preferable to the complete-case or missing-indicator method, they can also lead to bias (471), and it is important that all strategies for dealing with missing data are clearly specified and that the limitations of the chosen approach are made clear (472).

2.3.7. Analytical software

The analyses for this PhD thesis were carried out using SAS software, version 9.3 and 9.4 of the SAS System for Windows (SAS Institute Inc., Cary, NC, USA). Figures were created using Microsoft Office (Excel and PowerPoint) unless otherwise specified.

Chapter 3 . Patterns of Resistance Testing and Detected Drug Resistance in Europe

3.1. Introduction and Objectives

Due to the potential for drug resistance to limit the efficacy of specific ART drugs both on an individual and on a population level, it is important to monitor and study trends in the prevalence of drug resistance. The key issue when using routine data from clinical cohorts to estimate the prevalence of resistance is that not everyone who fails therapy with drug resistance will have a genotypic resistance test (GRT) result available (473). Genotypic resistance testing is recommended for all individuals in high income settings before the start of cART as well as after treatment failure (146,147). These recommendations are built on evidence from a number of clinical trials conducted primarily in the period 1999-2004 (Table 1.4, page 70). Since this time a number of new drugs and drug classes to use following virological failure (VF) have become available and patient populations have changed, both of which could limit the generalisability of these early trial findings. The evidence supporting the use of drug resistance testing as part of routine clinical care was questioned by Dunn et al. as early as 2004 (404), and it is likely that guidelines recommending universal resistance testing are not always followed in clinical practice. Although doubts surrounding the evidence supporting the use of routine GRT may be one of the reasons that clinical practice diverges from guidelines, a range of other factors are also likely to affect whether a GRT is done for a particular patient. These include the changing clinical context, a growing motivation to only use cost-effective or cost-saving interventions and limited availability of equipment and technical competency in low income settings.

Taken together, these factors have led to a changing and selected proportion of individuals being tested for drug resistance over time, which in turn complicates the choice of denominator for resistance prevalence analyses. A number of different approaches have been taken, which have resulted in different denominators being used in different studies. The possible denominators that could be used to estimate the prevalence of resistance in a population are:

i) the total number of genotypic tests (474,475)

ii) the total number of individuals with virological failure (VF) (476,477),

iii) the total number of individuals treated (478,479)

iv) the total population available irrespective of treatment history (480)

There have also been some attempts to estimate the resistance prevalence among those with no test result available, using multiple imputation (MI) (473) and data completion algorithms (481). All of the above approaches have advantages and limitations, and can be used to answer different questions (479). Using the whole treated population as the denominator gives an indication of the population burden of resistance, which is useful for public health planning (479). However, this requires comprehensive coverage of genotypic data, as it presumes that all those without a resistance test did not have resistance (473). Using the total number of individuals with VF as the denominator requires making a similar assumption: that all those who experience VF fail due to resistance, and not due to other factors such as poor adherence. Using the total number of GRTs as the denominator avoids making such assumptions at the cost of limited generalisability. Prevalence estimates using this denominator instead describe the clinical experience; that is, the probability of detecting resistance given that a test is done. Although the use of multiple imputation avoids making the strong assumptions outlined for the first two denominators to a certain extent, the accuracy of these estimates is still dependent on a number of assumptions, and the final estimates describe the burden of resistance in the population rather than the situation in clinics.

In this chapter I take approach (i), and use the total number of GRTs as the denominator in order to estimate the probability of detecting drug resistance over time in clinics. In order to provide a context for these estimates, I also describe trends in the probability of receiving a resistance test after VF, as well as predictors for receiving a resistance test.

The specific objectives of my analysis are:

1)

   a. To estimate the proportion of individuals who received a resistance test after VF per calendar year of follow-up (FU)
   b. To identify predictors of receiving a resistance test among those who experience VF

2)

   a. To estimate the proportion of individuals in EuroSIDA who have detected resistance per calendar year of FU.

b. To identify predictors of having detected resistance among those who experienced VF and had a resistance test result available

3.2. Literature Review

A review of the literature relating to these aims was conducted according to the principles and pre-defined search strategies outlined in Chapter 2. The selection of papers can be seen in Figure 3.1, the studies are described briefly in Table 3.1 and Table 3.2, and the findings summarised below.

3.2.1. Aim 1: Utilisation of resistance testing

In this literature review, eight papers reporting on the use of resistance testing were identified (Figure 3.1). Overall, these studies suggested a marked variation in the utilisation of resistance testing according to the setting and time-period studied. However, all studies found that the rate of resistance testing following VF is lower than the level which is recommended in current guidelines, which advises universal testing (Table 3.1). Pillay et al., in 2005, studied resistance prevalence in the UK in the period of 1999-2002. They found that around 30% of treatment switches were guided by a resistance test done within the 6 months preceding a switch (479). This is a relatively similar result to an analysis of data from the US HIV Outpatient Study (HOPS) cohort 1999-2005. Of 2,699 individuals who experienced virological failure in their analysis, only 915 (34%) had a GRT (482). A previous EuroSIDA analysis studying resistance testing up until 2008 also found relatively low overall rates of resistance testing (36.2%) (483). However, other cohort studies from the US (481,484) and Canada (475,485) have found higher rates of resistance testing following failure, with estimates ranging from 45%-82%.

In terms of time-trends, two studies identified for this literature review reported a decrease in the probability of receiving a resistance test after 2004 (483,485), three studies reported a stable proportion receiving a GRT over time in the time-periods 1999-2002 (479), 1999-2006 (484) and 1997-2011 (478). Two studies reported an increase in the proportion of individuals who received a resistance test over time, one in the US (481) 2000-2005 and one in Canada (1997-2010) (475).

Figure 3.1. Identification of articles for the literature review studying the use of resistance testing (Aim 1) and the prevalence of resistance (Aim 2)

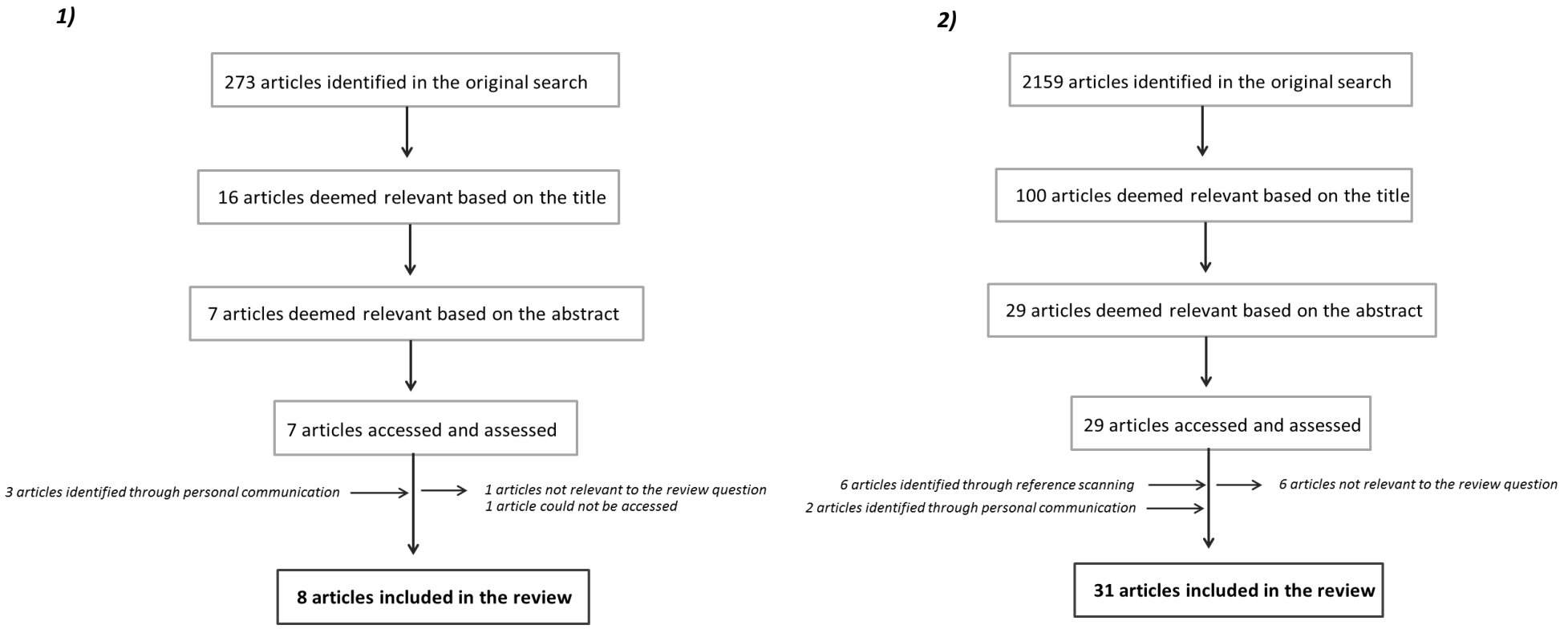


Table 3.1. Papers reporting on the use and predictors of receiving a GRT following VF (Aim 1)					
Author	**Year**	**Design and Setting**	**Study Size**	**Main Results**	**REF**
Cescon et al.	2014	Cohort study in Canada, 1997-2010	Not reported	The proportion of those with VF with a resistance test increased from 41% in 1997 to 82% in 2010.	(475)
Bontell et al.	2013	Prospective analyses of all patients receiving care in Sweden, 1997-2011	6,537	Found a relatively stable % received a GRT over time.	(478)
Eyawo et al.	2011	Cohort study in Canada (LISA), 2000 onwards	651	Of individuals eligible for resistance testing due to VF, a majority received a GRT. 37.6% did not receive a GRT. The odds of receiving a GRT were lower among individuals starting HAART in 2004 or later. The only other predictor of receiving a GRT was younger age.	(485)
Fox et al.	2011	Cohort study across Europe (EuroSIDA), 2002 onwards.	1,090	Overall, 36.2% of VFs were followed by a resistance test. This proportion decreased in more recent calendar years. Predictors of having a resistance test included geographical region, having had a previous resistance test and calendar year.	(483)
Abraham et al.	2011	Cohort Study in the US (NA-ACCORD), 2000-2005	9,289	The proportion of those with VF with a resistance test increased from 55% in 2000 to 62% in 2005.	(481)
Buchacz et al.	2010	Cohort Study in the US (HOPS), 1999-2006	3,995	Among patients with VF, 45% received a GRT at any point. This increased until 2003 and stabilised at around 30% per year of those eligible for a test afterwards. Predictors of having a resistance test included older age, female gender, lower CD4 count, higher VL and previous failure to at least two drug classes.	(484)
Palella et al.	2009	Cohort Study in the US (HOPS), 1999-2005	2,699	Of patients with VF, 915 (34%) had a GRT. Predictors of having a GRT were white race, MSM, having health insurance, a history of AIDS, low nadir CD4 and previous experience of mono/dual therapy.	(482)
Pillay et al.	2005	Cohort study in the UK, 1999-2002	4,218 with resistance: Denominator not reported	Up until 2002, only about 30% of treatment switches in the cohort were preceded by a resistance test within 6 months; although this proportion remained reasonably stable between 1999-2002	(479)

1. *REF=reference.*

Table 3.2. Papers reporting the prevalence and predictors of detected drug resistance following a successful GRT (Aim 2)						
Author	**Year**	**Design and Setting**	**Study Size**	**Denominator**	**Main Findings Summarised**	**REF**
Franzetti et al.	2014	Cohort study in Italy (ARCA cohort), 2003-2012	5,246	GRT	Described resistance prevalence over time and found a marked decrease over calendar time for all classes (NRTI: 79-40%; NNRTI:78-54%; PI-60-19%). Predictors of detecting resistance were: Gender, viral load (VL) at Failure, Previous VF and time.	(486)
Paquet et al.	2014	Prospective analysis of the commercial database from Monogram Biosciences in San Francisco, 2003-2012	62,397$	GRT	Described resistance prevalence in sequences submitted to the laboratory. Found a marked decrease in PI and NRTI resistance (PI: 44-21%; NRTI:79-57%), but an increase in NNRTI resistance (68-75%)	(480)
Cescon et al.	2014	Cohort study from BC, Canada, 1997-2010	Not reported	GRT	Studying resistance prevalence among patients tested with an unsuppressed viral load; prevalence decreased from 52%-18%.	(475)
Menezes et al.	2013	Cross-sectional study of prisoners in North Carolina, 2006-2010	367	ART	Representative surveillance study reporting an overall population prevalence of 28% resistance among inmates.	(487)
Bontell et al.	2013	Prospective analyses of all patients receiving care in Sweden, 1997-2011	6,537	ALL	Among all those treated for HIV, resistance to all drug three drug classes decreased over time. NRTI resistance decreased linearly, but resistance to NNRTI peaked in 2003-2004, decreased until 2007-2009 and increased in more recent calendar years. PI resistance peaked in 2001 and then decreased.	(478)
De Luca et al.	2013	Cohort collaboration in Western Europe (SEHERE database), 1997-2008	20,323	GRT	Reported an overall resistance prevalence of 80% among individuals tested for resistance, and a significant decrease over time for all classes (overall: 81%-71%).	(488)
Assoumou et al.	2012	Cross-sectional analysis of the ANRS cohort in France in 2009	506	GRT	Resistance found in 59% of genotyped patients. Compared to a previous analysis from 2004, this was significantly lower.	(489)

Miller et al.	2012	Repeated cross-sectional analysis of the commercial database from Monogram Biosciences in San Francisco, 2003 and 2010	107,231 $	GRT	Studied prevalence of individual NRTI mutations in sequences submitted to a laboratory. Found a decreased prevalence of several major NRTI resistance mutations in 2010 compared to 2004.	(481)
Abraham et al.	2011	Cohort study in the US (NA-ACCORD), 200-2005	9,289	ALL	Found a decreasing trends in those with any cumulative resistance when only using the existing data among those tested, however when imputing data for all those failing therapy they found a stable or possibly increasing trend. Also found that the prevalence of resistance in the entire cohort was much lower than in those with testing.	(474)
Bannister et al.	2011	Cross-sectional analysis using multiple imputation from the EuroSIDA cohort in 2008	6,498	ART	Estimated that the prevalence of drug resistance among 6498 patients receiving ART was 43% NRTI, 15% NNRTI and 25% PI resistance.	(473)
Gill et al.	2010	Cohort study from BC, Canada, 1997-2008	5,422	ART	Studied incidence rather than prevalence; found decreasing incidence of drug resistance among everyone receiving ART over time; from 1.73 cases with resistance per 100 PYFU to 0.13 cases per 100 PYFU.	(490)
Buchacz et al.	2009	Cohort study in the US (HIV Outpatient Study), 1999-2008	906	GRT	Some evidence of a decrease in resistance among those tested; from 88% in 1999 to 79% in 2008, although this was not significant in regression models (p=0.054).	(491)
Von Wyl et al.	2009	Cohort study in Switzerland (Swiss HIV Cohort Study), 1999-2007	8,064	ALL	The overall prevalence of resistance among ART experienced patients decreased form 57% in 1999 to 45% in 2007. The overall prevalence using those experiencing VF or exposed to mono/dual therapy as the denominator was 77%.	(492)
Audelin et al.	2009	Nationwide population based cohort in Denmark, 1998-2005	1,829	ART	Estimated incidence of resistance using all personyears of FU treated with HAART as the denominator. Found a decrease over time in the incidence of resistance to all 3 major drug classes.	(493)
Rumyantseva et al.	2009	Phylogenetic analysis from Russia	47	VF	Among individuals experiencing VF on HAART, only 45% had detected resistance.	(494)

Santoro et al.	2008	Cross-sectional analyses from a clinic in Bulgaria, 2002-2006	58	GRT	Among patients with ARV experience and a plasma sample that could be genotyped, 70.1% were found to have any drug resistance.	(495)
Vercauteren et al.	2008	Cohort study of a Portuguese resistance database, 2001-2006	3,394 $	GRT	In a total of 3394 sequences and using the total number of tests available as the denominator, the authors found a decrease in the prevalence of multi-class resistance; from 5.7 in 2001 to 2.7% in 2006 (however, as resistance was not carried forward in a cumulative manner this is referred to as incidence by the authors). Time on therapy was a significant predictor of multi class resistance.	(496)
McColll et al.	2008	Prospective analysis of the commercial database from Monogram Biosciences in San Francisco, 2003-2006	60,487 $	GRT	Studied trends in NRTI resistance among clinical isolates. Found down-ward time-trends in individual NRTI mutations.	(497)
Costagliola et al.	2007	Cross-sectional analysis of ANRS cohort data from France, 2004	488	VF	Of 488 patients with VF, 88% (ANRS) or 83% (Stanford) had detected resistance	(477)
Napravnik et al.	2007	Cross-sectional analysis of US cohort	1,724	ART	Of patients receiving some form of ART, 8% had resistance to all 3 drug classes. Predictors of TCR included nadir CD4, peak RNA, a previous AIDS defining condition, earlier calendar year of ART initiation, a history of non-HAART initial therapy and receiving a greater number of antiretroviral drugs.	(498)
Garcia-Guerrero et al.	2006	Cross-sectional analysis of prisoners in Spain	90	ART	38.6% of all treatment experienced prisoners were estimated to harbour resistance to any drug.	(499)
Tozzi et al.	2006	Cohort study at a hospital in Rome, Italy	602	VF	28.5% had class-wide NRTI resistance, 57.7% had class wide NNRTI resistance and 19.9% had PI class wide resistance. Time on treatment and a history of more previous regimens increased the odds of having class-wide resistance. AIDS was associated with an increased risk of class-wide resistance. Female gender and younger age decreased the odds of PI class wide resistance. Lower viral load was not associated with class-wide resistance to NNRTI or NRTI, but marginally associated with a lower risk of class-wide PI resistance.	(500)

Pillay et al.	2005	Cohort study in the UK (UK CHIC and HIV DRB), 1998-2002	4,218	GRT	Among patients tested for resistance, resistance prevalence remained fairly constant between 75% and 82%. Using all patients receiving treatment as the denominator, the prevalence of resistance to any drugs increased until 2000 and then stabilised at around 17%.	(479)
Phillips et al.	2005	Cohort study in the UK (UK CHIC and UK HIVDRB), 1998-2005	4,306	ART	The long-term probability of developing resistance among patients who started ART with 3 or more drugs was estimated at 26% by 6 years. There was no evidence that the probability of detecting resistance at 2 years after the start of ART had decreased during the study period. Predictors of resistance included younger age, starting ART with a high VL, having an unknown sexual risk group, having low CD4 count, previous AIDS and type of ART.	(501)
Richman et al.	2004	Cross-sectional analysis of cohort study in the US (HCSUS study), 1998	1,797	VF	Cross-sectional estimate of resistance prevalence in 1998 was 76.3% among those experiencing VF and 48% among all of those treated. Predictors of resistance were advanced HIV disease, Current Viral Load, CD4 nadir, Sexual Risk Group, Insurance Coverage and Education Level. In multivariable analyses only current viral load and CD4 nadir was associated with higher odds of detecting resistance.	(476)
Holodniy et al.	2004	Cross-sectional study in San Francisco, 2004	168	ART	Estimated that the prevalence of resistance among 168 treatment experienced patients was 36%. Predictors of resistance included race, health, insurance and ARV history.	(502)
Scott et al.	2004	Surveillance estimates from the UK, 1998-2000	300	ART	Estimated that the prevalence of any resistance among those treated for HIV in the UK was 27% in 1998, 41% in 1999 and 27% in 2000.	(503)
Tamalet et al.	2003	Prospective study of a hospital database in France, 1999-2002	7,799	GRT	Among genotyped patients, the overall prevalence of resistance was 78% NRTI, 39% NNRTI and 47% PI. NRTI trends were stable throughout the study period, whereas NNRTI and PI prevalence increased dramatically in the early 90s.	(504)
Gallego et al.	2002	Cross-sectional study in Spain, 2002	540	VF	Among patients who experienced failure, 79% had drug resistance; 77% NRTI, 53% PI and 42% PI.	(505)

Plettenberg et al.	2001	Cross-sectional analysis from a hospital in Hamburg, 2001	52	VF	Found a resistance prevalence of 80% among heavily pre-treated patients experiencing VF.	(506)

1. REF=reference.

$ Where possible, the number of individuals who contributed data was reported. When only the number of sequences was reported in the paper, this number is given an instead, highlighted with the symbol.

3.2.2. Predictors of receiving a resistance test

Predictors of receiving a resistance test varied across study settings, and included demographics such as age (484,485), gender (491) , and risk group (482), as well as clinical variables such as low current CD4 count, low CD4 nadir, and having a higher current VL (482,491). A history of mono/dual therapy or a previous resistance test was also reported to increase the probability of receiving resistance test in several studies (482,483,491).

3.2.3. Aim 2: prevalence of HIV drug resistance in high income settings

32 articles describing the prevalence of drug resistance in a variety of setting over different time-periods were identified (Figure 3.1). Of these, 13 used the proportion with a resistance test, 10 used the population of all treated individuals as the denominator and six used the proportion treated and experiencing failure, and. Finally, three reported comparative estimates using several different denominators (Table 3.2). Among the studies reporting it, the prevalence of resistance to any drug class varied from 59%-88% when using those tested for resistance as the denominator, 28-48% when using the population of treated individuals as the denominator and 45%-80% when using the population of treated individuals experiencing virological failure as the denominator. All studies that reported class-wide resistance found that NRTI resistance was more commonly detected than both NNRTI and PI resistance (Table 3.2).

Several studies reported on time-trends in resistance prevalence, with results differing somewhat depending on time-period, region of study and the denominator used. A recent study by Franzetti et al. analysing time-trends between 2003 and 2012 among individuals experiencing VF who were tested for resistance in Italy reported a marked decrease in the prevalence of resistance for both NRTI, NNRTI and PI resistance (486). This echoes the results of a 2013 study by De Luca et al., which studied trends in resistance prevalence among over 20,000 individuals with a genotype from the SEHERE collaboration in Western Europe. They reported a decrease over time for the prevalence of any resistance, as well as class-specific resistance and triple-class resistance (474). A decrease in resistance prevalence among those with a GRT has also been reported in Canada (475), France (488) and, looking at triple-class resistance specifically, in Portugal (496). Studies using the population of all treated individuals as the denominator have also reported a decrease in resistance prevalence over time in Canada (490), Denmark (493), Sweden (478), and Switzerland (492).

Some of these studies have reported a more marked resistance decrease after 2004 for NNRTI resistance, and a somewhat earlier peak for PI and NRTI resistance; with the highest prevalence estimates for these drug classes found around 2001 (478,507) . This is in

agreement with a number of studies conducted before 2002 that reported increasing or stable resistance prevalence for all drug classes before this time (479,503,504).

Data from the USA are more ambiguous. A recent study of resistance prevalence using sequences in the Monogram Biosciences commercial database in San Francisco found evidence of a decrease in NRTI and PI resistance, but an increase in NNRTI resistance (480). A study from the HIV outpatient study (HOPS) reported on an overall decrease in the prevalence of resistance, from 88% of those with a GRT in 1999 to 79% in 2008; however the statistical evidence supporting this decrease was of borderline statistical significance (p=0.054) (491). A study of resistance data in the US NA-ACCORD collaboration found a decreasing trend in resistance prevalence when using the individuals with a resistance test as the denominator, but when imputing data for all those with a missing GRT and using the number of people failing therapy as the denominator they found stable resistance trends (481).

3.2.4. Predictors of detecting drug resistance

Those studies that reported on predictors of drug resistance have found that gender (486), sexual risk group (474,501), VL at failure (476,486) or at the start of therapy (501), a previous VF (486), history of AIDS (476,501), low CD4 counts (476,501), type of ART (474,501) and subtype (474) were associated with the risk of detection of any resistance. Time spent on therapy (496,498,500), nadir CD4, peak VL, a history of AIDS and exposure to a greater number of antiretroviral drugs (498) have been associated with the detection of triple class resistance.

3.2.5. What the current analysis adds

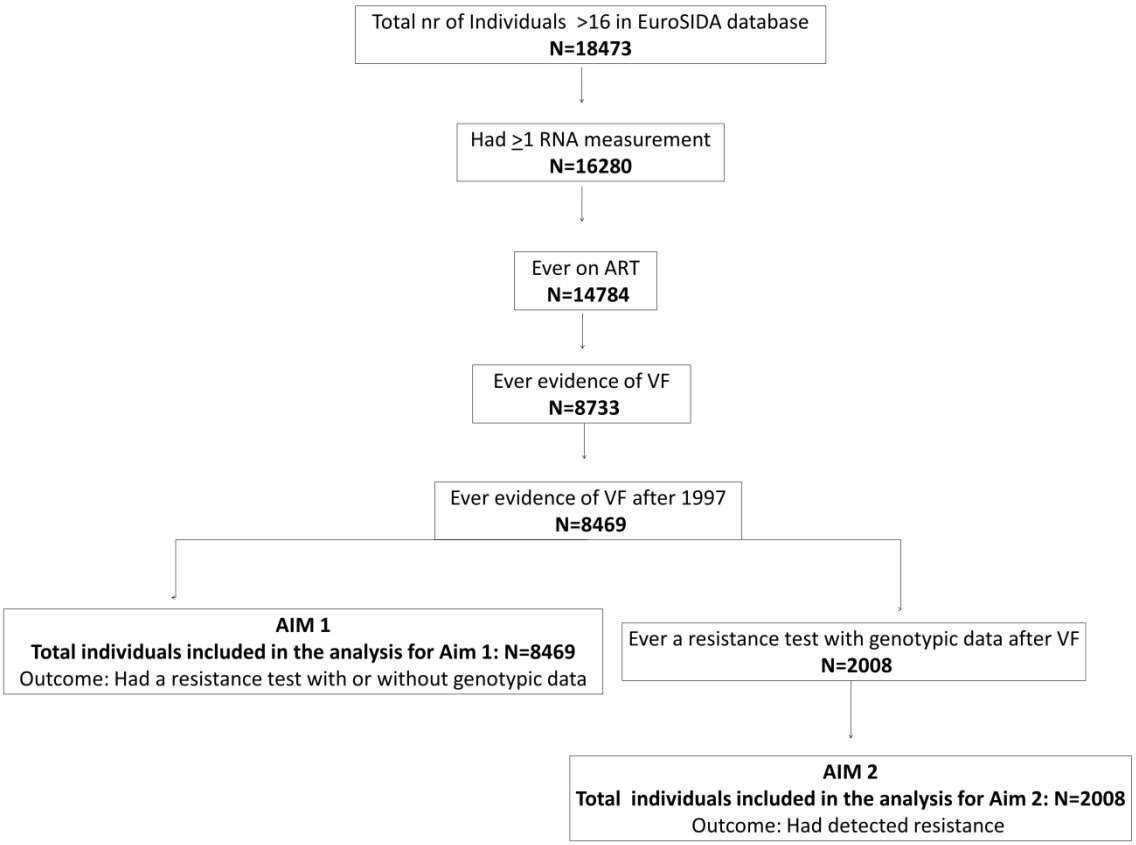
As shown in this literature review, previous analyses of resistance testing have only reported on time-trends up until 2008, and there is a lack of data on how clinicians use resistance testing in more recent calendar years. Although there is a lot of published data on time-trends in resistance, most of the reported data is either country-specific or, where data is reported on a European level, restricted to countries in Western Europe with data from other European regions more scarce. Comparative estimates of the risk of detecting resistance in geographical regions of Europe have also not been reported. EuroSIDA is the ideal setting for this type of analysis as people from different regions of Europe are enrolled following standardised inclusion criteria and monitoring procedures.

3.3. Methods

3.3.1. Inclusion Criteria

This analysis used the D36 update of the EuroSIDA database, which includes 18,473 individuals. The selection process for inclusion in the analysis can be seen in Figure 3.2**.**

Figure 3.2. Selection of individuals for inclusion in the study



For my first aim, looking at the utilisation of and predictors of resistance testing, I included individuals who had evidence of VF after the 1st of January 1997. I defined VF as having a single viral load (VL) measurement >500 copies/mL while on ART after at least 6 months of ART exposure. This is in line with the EACS recommendations at the time the analysis was conducted (2013) (147). Although this is the main definition, a large number of alternative definitions of VF were considered (described in detail in section 3.3.4, page 122). I consciously used a broad definition of VF for my primary analysis in order to maximise the number of individuals that we could include. I only considered VFs occurring after 1997, as this was when resistance data was first collected in EuroSIDA. For any given calendar year, a person was included in the denominator if they experienced VF in that year, and considered as having a resistance test associated with the VF (numerator) in that year if they had a resistance test no more than one month before or 12 months after the date of VF. If the same resistance test could be linked to more than one VF date in different calendar years, it was attributed to the VF date occurring closest to the test.

For the second aim of studying the prevalence and predictors of detected drug resistance, I included individuals who had a resistance test as well as genotypic results from this test. Individuals could contribute data for more than one calendar year if they had GRT and GRT results corresponding to multiple VF episodes, and were not excluded after they had their first VF or resistance test. The characteristics of the study population at the time of their first included VF were summarised using standard summary statistics and tests (section 2.3.2, page 95).

3.3.2. Resistance data

The structure of the EuroSIDA resistance data is described in detail in Chapter 2, Section 2.2.14. For all analyses, retrospective resistance tests - where the resistance results had been obtained from retrospectively testing samples stored in the EuroSIDA repository - were excluded. The rationale for excluding these tests was that it is difficult to establish whether these GRTs would also have been prescribed as part of routine practice. For the first aim, I therefore considered the date of all prospective resistance tests available in the EuroSIDA resistance database. I also considered a variable entered separately by the clinician on the case report form (CRF) which indicated the date of the latest resistance test. Where this did not overlap with the data in the EuroSIDA resistance database, I used this as an indicator that a separate resistance test had been performed. This latter variable did not have genotypic data associated with it, and these tests were therefore not counted for my second aim.

Resistance mutations were defined using the IAS-USA (2013) guidelines (335). Throughout the chapter, 'any resistance mutation' will refer to $\geq$1 detected IAS-USA resistance mutation to NRTI, NNRTI or PI drug classes, excluding minor PI mutations. Resistance to other drug classes, such as fusion or integrase inhibitors, is not routinely collected and was therefore not considered for this analysis. Integrase resistance is discussed in more detail in Chapter 4. As the aim of the analysis was not to estimate the current level of resistance likely to present in participants' body at the time of current GRT, but simply the probability to detect resistance at the specific point in time when the person was tested, resistance mutations detected in previous GRTs were not carried forward in a cumulative manner.

3.3.3. Statistical methods

The proportion of individuals with a resistance test following virological failure, and detected resistance after having a test, was plotted against calendar year with 95% confidence intervals (CI). Logistic regression models with generalised estimating equations (GEE) (508,509) were used to identify predictors and to test for changes in the prevalence of resistance testing and detected resistance. The GEE allowed me to account for non- independence of the statistical

units (people contributing to more than one year) in the model. The covariates and their categorisation is listed in Table 3.3.

Table 3.3 Categorisation of variables of interest		
Variable	**Categories**	**Time-updated**
Gender	Female; Male	No
Ethnicity	White, Non-white	No
Age	Continuous (per 10 years)	No
Mode of Transmission	MSM; PWID; Heterosexual; Other	No
Geographical Region	Southern, Central West, Northern, Central East, Eastern	No
Type of ARV used at VF	PI (boosted), PI (unboosted), NNRTI, PI and NNRTI, Neither PI or NNRTI	No
History of mono-dual therapy	Yes, No	No
VL at VF	<1000, 1000-10000, 10000-50000, >50000	No
CD4 at VF	Continuous, per 100 cells/mm^3	No
Number of previous resistance tests	None, 1, 2-4 ,>4	No
HIV Subtype[1]	B, Non-B, Unknown	No
Mono or dual therapy	Yes, No	No
Calendar Year of VF	97-98, 99-00, 01-02, 03-04, 05-06 07-08, 09-10, 11-12	No

1. Only in the model of resistance prevalence

3.3.4. Sensitivity, sub group and exploratory analyses

Several sensitivity analyses were performed using a number of definitions of virological failure, as well as number of sub-group and exploratory analyses. The main analysis was considered definition A.

Sensitivity analyses:

Definition B: Defining VF as at least two consecutive measures >500 instead of a single value, after at least 6 months on ART.

Definition C: Defining VF as a single value above the detection limit of the test used after at least 6 months on ART (cut-offs ranging from 20 copies/mL to 1000 copies/mL).

Definition D: Defining VF as a single VL >1000 (instead of >500) after at least 6 months on ART.

The rationale for these analyses was to test how much the results may vary depending on the type of viral load assay used as well as the exact definition of VF.

Sub-group and exploratory analyses:

Definition E: Primary VF definition (A), but also requiring an individual to have supressed VL to ≤500 before a subsequent failure could be included in the analysis.

Definition F: Primary VF definition (A), but also requiring that a VF was followed by a switch to a drug belonging to a class that was not included in the regimen started >6 months earlier.

The rationale for these analyses was to disentangle the effect that poor adherence, which may discourage a clinician to switch an individual's medication, may have on my results.

3.4. Results

3.4.1.Resistance testing

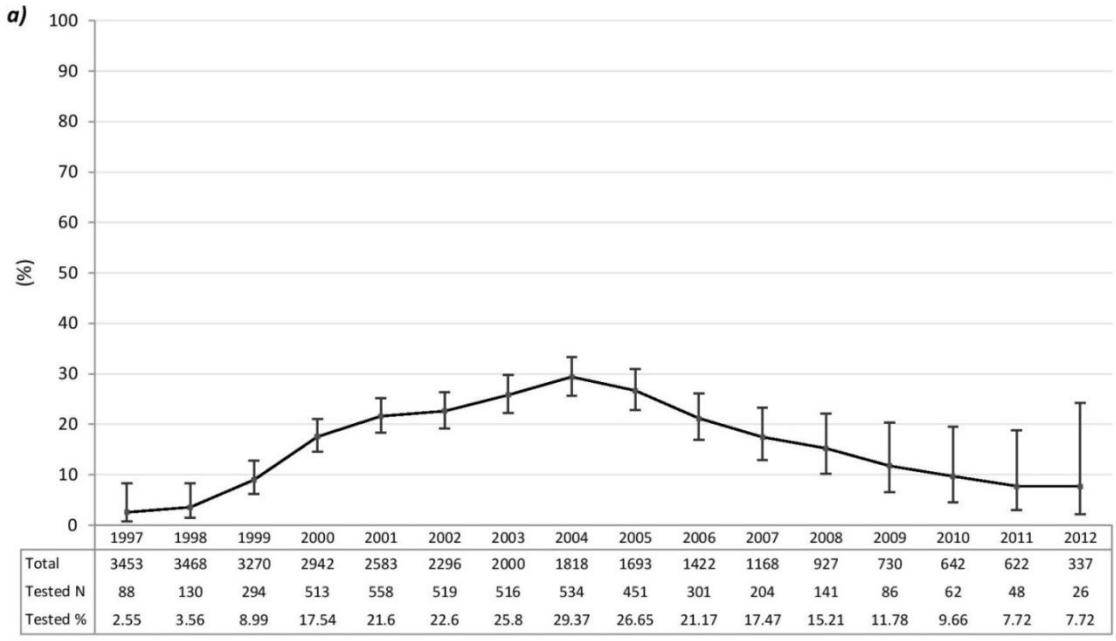
3.4.1.1. Trends in resistance testing

Of the 8,469 individuals experiencing virological failure, 2,676 (31.6%) were tested for resistance in at least one of the years in which they had evidence of VF. Among those who had a test, the median time between the date of VF and a resistance test was 0.7 months (IQR: 0-4 months; range: -1–12 months). Of those with at least one resistance test, 60.7% had one test, 23.5% had two tests, 8.5% had three tests and 7.3% had four or more resistance tests performed over the calendar years in which they contributed data. The proportion of individuals with a resistance test around the time of VF increased from just 2% in 1997 to 29% in 2004 and then declined back to 7.7% in 2012; Figure 3.3)

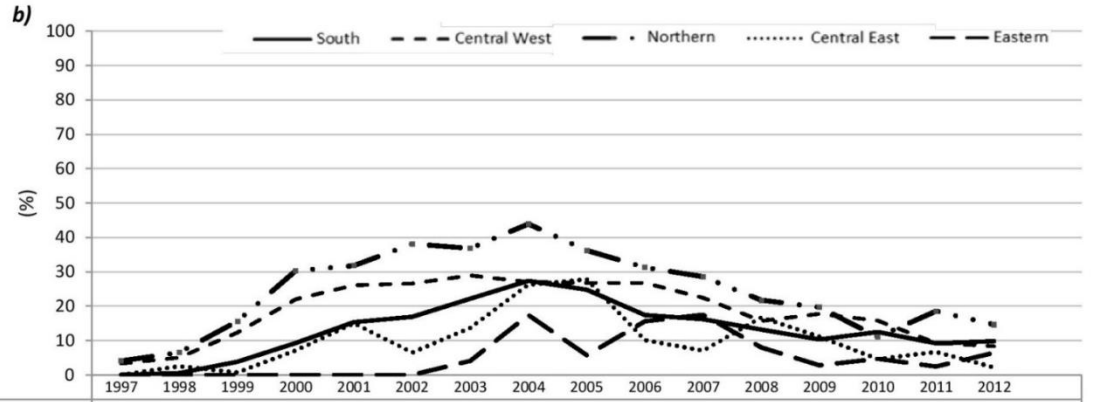
3.4.1.2. Factors associated with the probability of having a resistance test

The association between calendar year of VF and the probability of having a resistance test was confirmed in multivariable analysis (global p<.001, Table 3.4). Other predictors of having a resistance test around the date of VF included region: compared to Southern Europe, individuals were more likely to be tested for resistance in Northern Europe (aOR=2.15, 95%CI=1.96-2.36, p<.001) and Central Western Europe (aOR=1.66, 95%CI=1.51-1.82, p<.001). In contrast, individuals in Eastern Europe were less likely to be tested for resistance (aOR=0.72, 95%CI=0.55-0.94, p=0.02) compared to individuals in Southern Europe. Due to a small number of individuals being tested for resistance per region and per calendar year, I could not perform a formal interaction test as mathematical execution of the test was prevented by a lack of data. However, plotting the time-trends by region (Figure 3.3) showed that both the rise and decline in resistance testing following VF was somewhat more marked in Northern, Central Western, and Southern Europe when compared to Central Eastern and Eastern Europe. It is important to note that these trends are hard to interpret due to the small sample size within the subgroups. As expected, individuals with VL-levels of 1,000-10,000 copies/mL at VF were more likely to have a resistance test compared to individuals with lower viral loads (aOR=2.10, 95%CI=1.86-2.37, p<.001 compared to 500-999 copies/mL, Table 3.4).

Figure 3.3. Proportion of individuals with a resistance test following VF overall (a) and by region (b)



	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012
Total	3453	3468	3270	2942	2583	2296	2000	1818	1693	1422	1168	927	730	642	622	337
Tested N	88	130	294	513	558	519	516	534	451	301	204	141	86	62	48	26
Tested %	2.55	3.56	8.99	17.54	21.6	22.6	25.8	29.37	26.65	21.17	17.47	15.21	11.78	9.66	7.72	7.72



	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012
South N	0	7	50	112	159	156	168	206	173	100	57	40	22	18	12	8
South %	0	0.5	3.8	9.3	15.3	16.9	22.2	27.4	24.8	17.4	16.2	13.2	10.3	12.5	9.2	9.8
Central West N	38	54	116	176	185	159	158	123	106	91	59	28	23	16	8	4
Central West %	3.3	5	12.3	22	26.1	26.6	28.9	27	26.8	26.8	22.4	15.7	17.8	15.8	9.3	8.3
Northern N	50	66	127	209	181	188	149	143	108	70	59	34	23	9	12	6
Northern %	4.1	6.5	15.6	30.3	31.8	38.1	36.8	43.9	36.2	31.3	28.6	21.7	19.7	11	18.5	14.6
Central East N	0	3	1	13	29	13	27	51	49	15	10	18	10	5	6	1
Central East %	0	2.5	0.7	7.1	15	6.5	13.7	26.2	27.8	10.1	7	16.7	11.1	4.6	6.7	2.2
Eastern N	0	0	0	0	0	0	2	8	3	12	14	12	4	8	5	7
Eastern %	0	0	0	0	0	0	4.1	17.4	5.7	15.6	17.5	8	2.8	4.7	2.4	6.4

Table 3.4. Factors associated with having a resistance test after VF

		Unadjusted		Adjusted[1,2]		
		OR (95% CI)	**p-value**	**OR (95% CI)**	**p-value**	**Global p-value**
Age, per 10 year increase		1.26 (1.22 - 1.31)	<.001	1.03 (0.99 - 1.08)	0.10	0.12
CD4 at failure, per 100 increase[2]		0.99 (0.97 - 1.00)	0.09	1.01 (1.00 - 1.03)	0.07	0.08
Gender	*Male*	*1.00*		*1.00*		0.75
	Female	0.86 (0.79 - 0.95)	0.002	0.98 (0.89 - 1.08)	0.75	
Ethnic Group	*White*	*1.00*		*1.00*		
	Non-white	1.16 (1.04 - 1.30)	0.01	0.83 (0.75 - 0.92)	<.001	<.001
Risk Group	*MSM*	*1.00*		*1.00*		
	PWID	0.68 (0.61 - 0.75)	<.001	0.91 (0.82 - 1.00)	0.05	0.03
	Heterosexual	0.88 (0.80 - 0.97)	0.01	1.03 (0.93 - 1.14)	0.54	
	Other	1.03 (0.89 - 1.20)	0.68	1.10 (0.96 - 1.26)	0.19	
Region	*South*	*1.00*		*1.00*		<.001
	Central West	1.56 (1.40 - 1.72)	<.001	1.66 (1.51 - 1.82)	<.001	
	North	2.03 (1.84 - 2.24)	<.001	2.15 (1.96 - 2.36)	<.001	
	Central East	0.98 (0.84 - 1.15)	0.82	0.94 (0.81 - 1.09)	0.41	
	East	0.55 (0.42 - 0.71)	<.001	0.72 (0.55 - 0.94)	0.02	
ARVs used at failure	*PI (boosted)*	*1.00*		*1.00*		0.01
	PI (unboosted)	0.67 (0.58 - 0.76)	<.001	0.92 (0.80 - 1.05)	0.202	
	NNRTI	1.08 (0.91 - 1.29)	0.36	1.11 (0.93 - 1.33)	0.24	
	PI and NNRTI	1.76 (1.58 - 1.96)	<.001	0.96 (0.86 - 1.07)	0.49	
	Neither PI or NNRTI	0.50 (0.60 - 0.42)	<.001	0.77 (0.65 - 0.91)	0.003	
History of mono/dual therapy	*No*	*1.00*		*1.00*		0.48

	Yes	1.27 (1.15 - 1.40)	<.001	0.96 (0.86 - 1.07)	0.47	
RNA at failure	*<1000*	*1.00*		*1.00*		<.001
	1000-10000	1.91 (1.71 - 2.14)	<.001	2.10 (1.86 - 2.37)	<.001	
	10000-50000	2.38 (2.12 - 2.67)	<.001	2.74 (2.43 - 3.10)	<.001	
	>50000	2.48 (2.18 - 2.82)	<.001	2.68 (2.34 - 3.07)	<.001	
Previous Resistance Test	*None*	*1.00*		*1.00*		<.001
	1	10.32 (9.26 - 11.51)	<.001	6.26 (5.51 - 7.12)	<.001	
	2-4	11.19 (9.72 - 12.89)	<.001	6.76 (5.75 - 7.93)	<.001	
	>4	14.85 (9.59 - 22.99)	<.001	11.23 (7.04 - 17.92)	<.001	
Previous Virological failure	*No*	*1.00*		*1.00*		0.002
	Yes	3.16 (2.88 - 3.46)	<.001	1.19 (1.06 - 1.33)	0.003	
Calendar Year	*97-98*	0.08 (0.07 - 0.10)	<.001	0.11 (0.09 - 0.14)	<.001	<.001
	99-00	0.39 (0.36 - 0.44)	<.001	0.49 (0.43 - 0.55)	<.001	
	01-02	0.75 (0.68 - 0.82)	<.001	0.85 (0.76 - 0.95)	0.003	
	03-04	*1.00*		*1.00*		
	05-06	0.84 (0.75 - 0.94)	0.002	0.77 (0.68 - 0.89)	<.001	
	07-08	0.52 (0.45 - 0.60)	<.001	0.46 (0.39 - 0.54)	<.001	
	09-10	0.32 (0.26 - 0.39)	<.001	0.33 (0.27 - 0.41)	<.001	
	11-12	0.22 (0.17 - 0.28)	<.001	0.25 (0.19 - 0.32)	<.001	

1. The multivariable model is adjusted for all the variables listed in the table.

2. 26 individuals were excluded from the model with CD4 count as a covariate as well as the multivariable model due to missing CD4 counts.

Other factors associated with the probability of having a resistance test included ethnicity, with individuals of non-white ethnicity being less likely to receive a resistance test (aOR=0.83, 95%CI=0.75-0.92, p<0.001) compared to individuals of white ethnicity. There was also some evidence that people who inject drugs (PWID) were less likely to receive a resistance test compared to men who have sex with men (MSM; aOR=0.91, 95%CI=0.82-1.00, p=0.05). Individuals with a history of mono or dual therapy before starting cART were more likely to receive a resistance test in univariable analyses compared to those who those who had no history of mono or dual therapy (unadjusted odds ratio [uOR]=1.27, 95%CI=1.15-1.40, p<0.001), but not after adjustment for previous VFs (aOR=0.96, 95%CI=0.86-1.07, p=0.47) which appeared to be the main confounder for this association. It is possible that there is some overlap between what these variables are trying to capture (exposure to previously inadequate treatment), and a phi correlation test showed a significant but moderate correlation between the two variables (phi=0.22, p<0.0001). However, collinearity did not appear to be a problem for the overall model (all variance inflation factors [VIF] < 1.5). Individuals who had previously experienced a VF were more likely to have a resistance test (p=0.003) as were individuals with a large number of prior resistance tests (p<0.001). The type of ARV therapy used at failure was also associated with the odds of receiving a resistance test, with individuals currently receiving aPI or an NNRTI sparing cART regimens being less likely to receive a resistance test (aOR=0.77, 95%CI=0.65-0.91 compared to people using boosted PI cART). I did not find any strong evidence supporting an association between gender or age and receiving a resistance test (p=0.12 and 0=0.88 respectively, Table 3.4).

3.4.2. Detected drug resistance

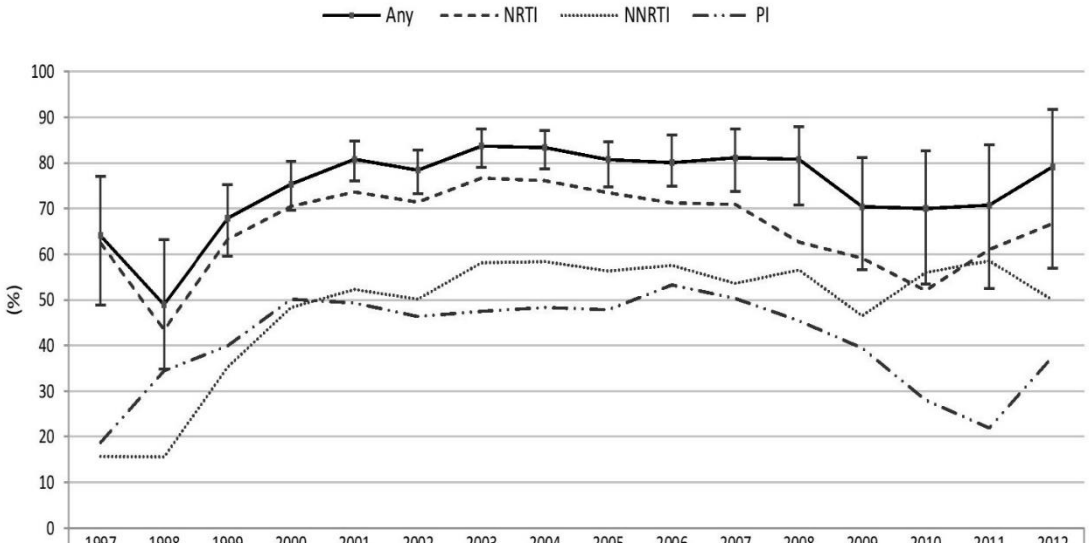
3.4.2.1.Trends in detected drug resistance

In total, drug resistance was detected in 2,431 (77.9%) of the 3119 resistance test results included. The prevalence of mutations with a prevalence of >10% is shown in Figure 3.5. Overall, NRTI resistance was most commonly detected (in 70.3% of tests), followed by NNRTI (51.6%) and PI (46.1%) resistance. The most commonly detected individual mutations were M184V (46.3%, associated with NRTI resistance), K103NS (23.4%, associated with NNRTI resistance) and L90M (26.8%, associated with PI resistance).

Changes in the proportion of individuals with detected drug resistance by calendar year can be seen in Figure 3.4, both overall and after stratification by drug class. Univariable models indicated that calendar year was associated with the risk of detection of drug resistance (global p <.001). This trend was not linear, and the prevalence appeared to increase until 2003-2004, followed by a tendency towards a decrease. In 1997, just less than two thirds of the population had detected resistance, and this was somewhat higher (84%) by 2003. In 2012, an

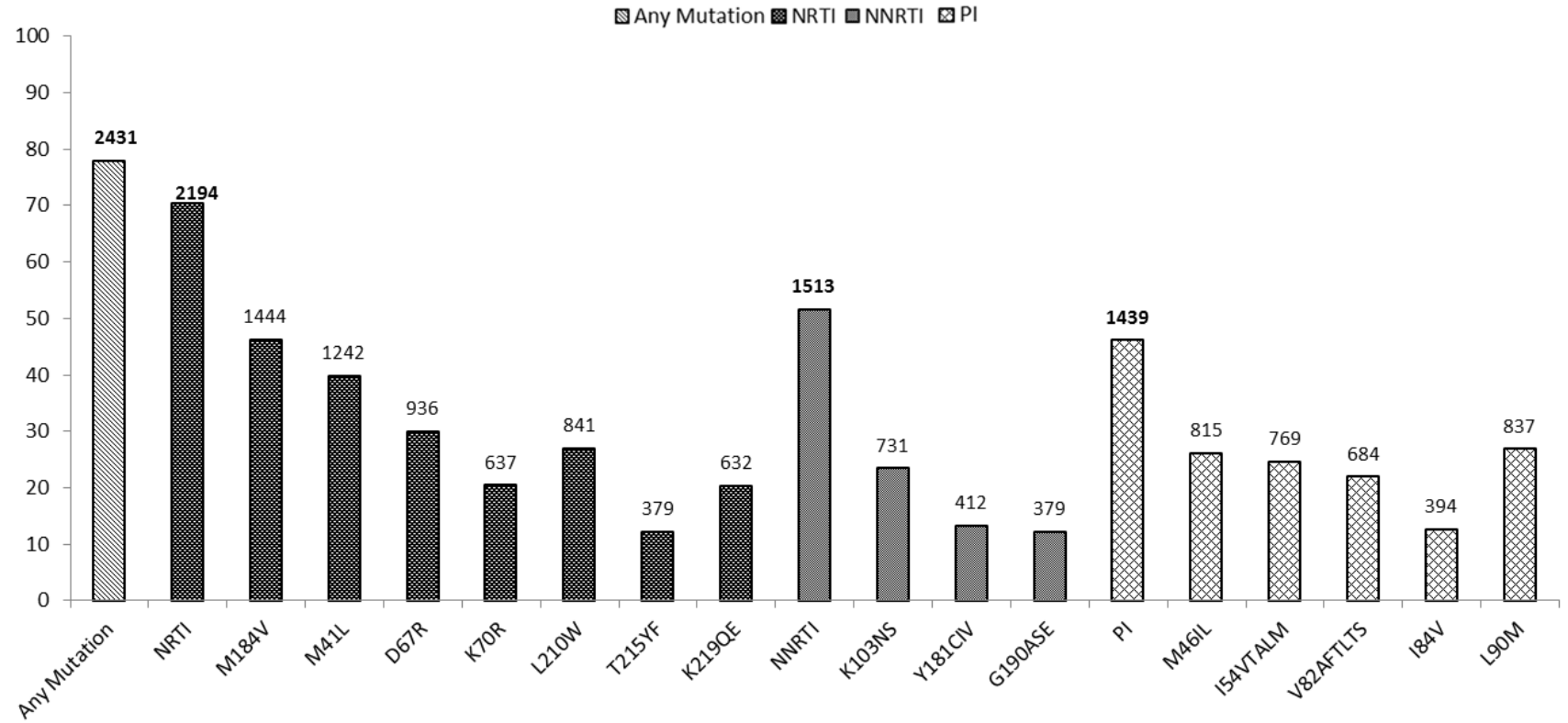
estimated 79% of individuals had detected drug resistance. Comparing specific time periods in univariable models, I found strong evidence (all p<.01) that the odds of detecting resistance were lower in the years of 97-98, 99-00 and 09-10 compared to 03-04 (Table 3.5).

Figure 3.4. Prevalence of detected drug resistance among individuals with GRT following VF, by drug class



	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012
Any	64.06	48.89	67.88	75.38	80.8	78.44	83.71	83.33	80.7	80.06	81.11	80.8	70.42	70	70.73	79.16
NRTI	62.5	43.33	63.21	70.46	73.6	71.43	76.69	76.11	73.42	71.24	70.86	62.63	59.15	52	60.98	66.66
NNRTI	15.63	15.56	35.23	48.31	52.26	50.13	58.15	58.33	56.33	57.51	53.58	56.57	46.48	56	58.54	50
PI	18.75	34.44	39.9	50.15	49.33	46.36	47.47	48.33	47.78	53.22	50.33	45.45	39.44	28	21.95	37.5

Figure 3.5. Prevalence of detected drug resistance mutations (with a prevalence of >10%)[1]



1. Absolute numbers in each category are shown on top of the bars.

3.4.2.2. Factors associated with the risk of detecting drug resistance

The odds of detecting drug resistance varied by calendar time also in multivariable models (global p<.001), with the odds of detecting any resistance, again, being lower before and after 2003-2004 (Table 3.5). Individuals were less likely to have resistance detected in Northern (aOR=0.29, 95%CI=0.21-0.39, p<.001) and Central Eastern Europe (aOR=0.47, 95%CI=0.29-0.76, p=0.002) compared to Southern Europe.

A number of other factors were independently associated with the risk of resistance detection. Individuals with a history of mono/dual therapy before starting cART were more likely to have detected drug resistance (aOR=1.54 vs. those who started cART from ART-naive, 95%CI=1.14-2.08, p=.007), as were individuals who had previously experienced virological failure (aOR=1.85 vs. those who experienced VF for the first time, 95%CI=1.40-2.45 p<.001). Individuals with RNA levels between 1,000 and 10,000 copies/mL were more likely to have detected resistance (aOR=1.63, compared to individuals with RNA levels less than 1,000 95%CI=1.19-2.23, p=0.002). Interestingly, individuals with very high RNA levels (>50,000 copies/mL) were not significantly more likely to have detected resistance (aOR=1.20, 95%CI=0.84-1.72) compared to individuals with RNA levels less than 1,000. There was some weak evidence that women and people who inject drugs were less likely to have detected drug resistance given that a test was done compared to men and men who have sex with men respectively (aOR=0.74, 95%CI=0.55-0.98, p=0.04 and aOR=0.69, 95%CI=0.51-0.95, p=0.02, respectively).

Table 3.5. Factors associated with having detected resistance (any class)

		Unadjusted		Adjusted[1,2]		
		OR (95% CI)	**p-value**	**OR (95% CI)**	**p-value**	**Global p-value**
Age	*<35*	*1.00*		*1.00*		
	35-40	1.35 (1.01 - 1.80)	0.04	1.13 (0.82 - 1.55)	0.45	0.95
	45-50	1.47 (1.09 - 1.99)	0.01	1.06 (0.75 - 1.50)	0.73	
	50-55	1.41 (1.05 - 1.90)	0.02	1.11 (0.78 - 1.57)	0.57	
	>55	1.43 (0.99 - 2.07)	0.05	1.13 (0.75 - 1.72)	0.55	
Gender	*Male*	*1.00*		*1.00*		
	Female	0.75 (0.60 - 0.94)	0.01	0.74 (0.55 - 0.98)	0.04	0.04
Ethnic Group	*White*	*1.00*		*1.00*		
	Non-white	0.87 (0.67 - 1.12)	0.28	0.94 (0.70 - 1.26)	0.66	0.67
Risk Group	*MSM*	*1.00*		*1.00*		
	IDU	0.90 (0.69 - 1.17)	0.43	0.69 (0.51 - 0.95)	0.02	0.04
	Heterosexual	1.04 (0.82 - 1.32)	0.75	1.12 (0.82 - 1.53)	0.48	
	Other	1.05 (0.71 - 1.56)	0.79	0.93 (0.60 - 1.44)	0.76	
Region	*South*	*1.00*				
	Central West	0.74 (0.55 - 1.00)	0.05	0.81 (0.58 - 1.13)	0.22	<.0001
	North	0.28 (0.21 - 0.37)	<.001	0.29 (0.21 - 0.39)	<.001	
	Central East	0.43 (0.27 - 0.69)	<.001	0.47 (0.29 - 0.76)	0.002	
	East	0.63 (0.31 - 1.29)	0.21	1.03 (0.46 - 2.32)	0.94	
History of mono/dual therapy	*No*	*1.00*				
	Yes	1.63 (1.31 - 2.01)	<.001	1.54 (1.14 - 2.08)	0.004	0.007

Subtype	*B*	*1.00*				
	Non-B	0.78 (0.59 - 1.04)	0.09	0.80 (0.57 - 1.10)	0.17	0.23
	Unknown	1.07 (0.84 - 1.36)	0.60	0.83 (0.63 - 1.09)	0.18	
RNA at failure	*<1000*	*1.00*				
	1000-10000	1.67 (1.25 - 2.24)	<.001	1.63 (1.19 - 2.23)	0.002	0.002
	10000-50000	1.86 (1.39 - 2.51)	<.001	1.68 (1.22 - 2.31)	0.002	
	>50000	1.35 (0.98 - 1.85)	0.06	1.20 (0.84 - 1.72)	0.32	
CD4 at failure, per 100 increase[2]		0.98 (0.95 - 1.02)	0.39	0.95 (0.91 - 0.99)	0.01	0.02
Previous Resistance Test	*No*	*1.00*				
	Yes	1.07 (0.89 - 1.28)	0.46	0.88 (0.71 - 1.09)	0.23	0.23
Previous Virological failure	*No*	*1.00*				
	Yes	2.53 (2.02 - 3.15)	<.001	1.85 (1.40 - 2.45)	<.001	<.0001
Calendar Year	*97-98*	0.24 (0.16 - 0.36)	<.001	0.31 (0.19 - 0.49)	<.001	<.0001
	99-00	0.52 (0.39 - 0.69)	<.001	0.47 (0.34 - 0.65)	<.001	
	01-02	0.77 (0.60 - 1.00)	0.05	0.75 (0.57 - 0.99)	0.04	
	03-04	*1.00*				
	05-06	0.82 (0.62 - 1.08)	0.15	0.73 (0.55 - 0.98)	0.03	
	07-08	0.85 (0.59 - 1.23)	0.39	0.90 (0.60 - 1.35)	0.61	
	09-10	0.47 (0.30 - 0.72)	<.001	0.49 (0.30 - 0.79)	0.003	
	11-12	0.56 (0.31 - 0.99)	0.05	0.68 (0.36 - 1.27)	0.23	

1. The multivariable model is adjusted for all the variables listed in the table.

2. 3 individuals were excluded from the model with CD4 count as a covariate as well as the multivariable model due to missing CD4 counts.

3.4.3. Sensitivity, sub-group and exploratory analyses

The estimates of the prevalence of resistance tests following VF and resistance from the sensitivity, subgroup and exploratory analyses are shown in Table 3.6**.** Briefly, the proportion of individuals with a resistance test was higher when using stricter criteria to define virological failure, but remained below 50%. This included insisting on a confirmatory value >500 copies (37%) and when defining VF as only episodes followed by a switch in regimen (47%). The proportion of resistance tests with detected drug resistance remained reasonably stable regardless of the definitions of virological failure used.

Multivariable results from these analyses are shown for two variables of interest: region and calendar year, in Table 3.7-Table 3.10 below. These results from the sensitivity analyses (Definitions B-D) were consistent with the primary analysis for both the model of resistance testing and the model of resistance prevalence. Although some findings did not reach statistical significance as the sample size decreased, the odds ratios remained reasonably stable, confirming the geographical and time-trends reported for both resistance testing and the prevalence of detected resistance. This was also the case for the subgroup and exploratory analyses.

Table 3.6. Prevalence of testing and resistance in sensitivity, sub-group and exploratory analyses

	Definition A[1]	**Definition B[2]**	**Definition C[3]**	**Definition D[4]**	**Definition E[5]**	**Definition**
	N *(%)*	**N *(%)***	**N *(%)***	**N *(%)***	**N *(%)***	**N *(%)***
Failing	8468	6017	9661	7828	5748	3776
Resistance test/individual	2676 (*31.6)*	2204 (*36.6)*	2210 (22.9)	2547 (3*2.5)*	1863 *(32.4)*	1767 *(46.8)*
Detected resistance/test	2431 (*77.9)*	2085 (*81.4)*	1899 (76.8)	2309 *(78.8)*	1788 *(76.5)*	737 *(83.6)*

1. Primary definitions.

2. Defining VF as at least 2 consecutive measures >500 instead of a single value, after at least 6 months on ART.

3. Defining VF as a single value above the detection limit of the test used after at least 6 months on ART.

4. Defining VF as a single VL >1000 (instead of >500) after at least 6 months on ART.

5. Defining failure as a single viral load >500, requiring an individual to supress to <500 before a subsequent failure was included in the analysis.

6. Defining VF as a single viral load >500 followed by a switch to a drug belonging to a class that was not included in the regimen started >6 months earlier.

Table 3.7. Region and calendar year associations with having a resistance test in sensitivity analyses (Definition B-D)[1]

		Definition A[2]		Definition B[3]		Definition C[4]		Definition D[5]	
		aOR (95% CI)	**Global P**	**aOR (95% CI)**	**Global P**	**aOR (95% CI)**	**Global P**	**aOR (95% CI)**	**Global P**
Region	*South*	*1.00*		*1.00*		*1.00*		*1.00*	
	Central West	1.66 (1.51 - 1.82)	<.0001	1.58 (1.42 - 1.76)	<.0001	1.55 (1.38 - 1.73)	<.001	1.61 (1.46 - 1.77)	<.001
	North	2.15 (1.96 - 2.36)		2.18 (1.96 - 2.43)		2.16 (1.94 - 2.41)		2.09 (1.90 - 2.31)	
	Central East	0.94 (0.81 - 1.09)		0.96 (0.82 - 1.14)		0.73 (0.62 - 0.87)		0.98 (0.85 - 1.13)	
	East	0.72 (0.55 - 0.94)		0.63 (0.46 - 0.86)		0.67 (0.50 - 0.89)		0.72 (0.55 - 0.95)	
Calendar Year	*97-98*	0.11 (0.09 - 0.14)	<.0001	0.10 (0.08 - 0.12)	<.0001	0.09 (0.06 - 0.14)	<.001	0.11 (0.09-0.14)	<.001
	99-00	0.49 (0.43 - 0.55)		0.48 (0.42 - 0.55)		0.43 (0.37 - 0.50)		0.50 (0.44-0.57)	
	01-02	0.85 (0.76 - 0.95)		0.84 (0.74 - 0.95)		0.83 (0.74 - 0.93)		0.86 (0.77-0.97)	
	03-04	*1.00*		*1.00*		*1.00*		*1.00*	
	05-06	0.77 (0.68 - 0.89)		0.74 (0.63 - 0.86)		0.79 (0.69 - 0.89)		0.79 (0.69-0.91)	
	07-08	0.46 (0.39 - 0.54)		0.48 (0.39 - 0.58)		0.43 (0.37 - 0.50)		0.45 (0.38-0.53)	
	09-10	0.33 (0.27 - 0.41)		0.34 (0.26 - 0.45)		0.32 (0.26 - 0.39)		0.31 (0.25-0.39)	
	11-12	0.25 (0.19 - 0.32)		0.33 (0.21 - 0.49)		0.21 (0.17 - 0.27)		0.26 (0.19-0.34)	

1. *Adjusted for age, gender, ethnicity, mode of HIV transmission, previous history of mono/dual therapy use, number of available previous resistance tests, CD4 count at failure, VL at failure and previous history of VF.*
2. *Primary definition.*
3. *Defining VF as at least 2 consecutive measures >500 instead of a single value, after at least 6 months on ART.*
4. *Defining VF as a single value above the detection limit of the test used after at least 6 months on ART.*
5. *Defining VF as a single VL >1000 (instead of >500) after at least 6 months on ART.*

Table 3.8. Region and calendar year associations with having a resistance test in sensitivity analyses (Definition E-F)[1]

		Definition A[2]		Definition E[3]		Definition F[4]	
		aOR (95% CI)	**Global P**	**aOR (95% CI)**	**Global P**	**aOR (95% CI)**	**Global P**
Region	*South*	*1.00*	<.0001	*1.00*	<.0001	*1.00*	<.0001
	Central West	1.66 (1.51 - 1.82)		1.62 (1.45 - 1.81)		1.40 (1.17 - 1.66)	
	North	2.15 (1.96 - 2.36)		2.12 (1.91 - 2.36)		1.78 (1.48 - 2.12)	
	Central East	0.94 (0.81 - 1.09)		0.91 (0.76 - 1.09)		0.73 (0.55 - 0.96)	
	East	0.72 (0.55 - 0.94)		0.59 (0.42 - 0.84)		0.74 (0.47 - 1.18)	
Calendar Year	*97-98*	0.11 (0.09 - 0.14)	<.0001	0.09 (0.08 - 0.12)	<.0001	0.07 (0.05 - 0.10)	<.0001
	99-00	0.49 (0.43 - 0.55)		0.50 (0.44 - 0.58)		0.40 (0.33 - 0.49)	
	01-02	0.85 (0.76 - 0.95)		0.86 (0.76 - 0.97)		0.75 (0.62 - 0.92)	
	03-04	*1.00*		*1.00*		*1.00*	
	05-06	0.77 (0.68 - 0.89)		0.77 (0.67 - 0.88)		0.73 (0.57 - 0.94)	
	07-08	0.46 (0.39 - 0.54)		0.48 (0.40 - 0.57)		0.42 (0.30 - 0.57)	
	09-10	0.33 (0.27 - 0.41)		0.35 (0.28 - 0.44)		0.31 (0.21 - 0.45)	
	11-12	0.25 (0.19 - 0.32)		0.29 (0.21 - 0.41)		0.420.25 - 0.71)	

1. *Adjusted for age, gender, ethnicity, mode of HIV transmission, previous history of mono/dual therapy use, number of available previous resistance tests, CD4 count at failure, VL at failure and history of previous VF*
2. *Primary definition.*
3. *Defining failure as a single viral load >500, requiring an individual to supress to <500 before a subsequent failure was included in the analysis.*
4. *Defining VF as a single viral load >500 followed by a switch to a drug belonging to a class that was not included in the regimen started >6 months earlier*

Table 3.9. Region and calendar year associations with the risk of detecting resistance according to sensitivity analyses (Definition B-D)[1]

		Definition A[2]		Definition B[3]		Definition C[4]		Definition D[5]	
		aOR (95% CI)	Global P	aOR (95% CI)	Global P	aOR (95% CI)	Global P	aOR (95% CI)	Global P
Region	*South*	*1.00*		*1.00*		*1.00*		*1.00*	
	Central West	0.81 (0.58 - 1.13)	<.0001	0.92 (0.62 - 1.37)	<.0001	0.57 (0.40 - 0.81)	<0.001	0.87 (0.62-1.22)	<0.001
	North	0.29 (0.21 - 0.39)		0.27 (0.19 - 0.39)		0.27 (0.19 - 0.38)		0.29 (0.21-0.40)	
	Central East	0.47 (0.29 - 0.76)		0.52 (0.29 - 0.94)		0.44 (0.26 - 0.74)		0.51 (0.31-0.85)	
	East	1.03 (0.46 - 2.32)		1.09 (0.38 - 3.14)		1.33 (0.58 - 3.07)		1.01 (0.46-2.3)	
Calendar Year	*97-98*	0.31 (0.19 - 0.49)	<.0001	0.31 (0.18 - 0.54)	<.0001	0.45 (0.18 - 1.13)	0.13	0.28 (0.17-0.46)	<0.001
	99-00	0.47 (0.34 - 0.65)		0.46 (0.31 - 0.67)		0.64 (0.42 - 0.95)		0.47 (0.33-0.66)	
	01-02	0.75 (0.57 - 0.99)		0.77 (0.56 - 1.07)		0.85 (0.63 - 1.15)		0.76 (0.56-1.01)	
	03-04	*1.00*		*1.00*		*1.00*		*1.00*	
	05-06	0.73 (0.55 - 0.98)		0.86 (0.59 - 1.25)		0.77 (0.58 - 1.02)		0.70 (0.50-0.93)	
	07-08	0.90 (0.60 - 1.35)		1.25 (0.71 - 2.20)		0.82 (0.55 - 1.22)		0.85 (0.55-1.31)	
	09-10	0.49 (0.30 - 0.79)		0.36 (0.20 - 0.63)		0.59 (0.38 - 0.92)		0.34 (0.22-0.61)	
	11-12	0.68 (0.36 - 1.27)		1.25 (0.47 - 3.37)		0.66 (0.38 - 1.14)		0.59 (0.31-1.13)	

1. *Adjusted for age, gender, ethnicity, mode of HIV transmission, previous use of mono/dual therapy, number of available previous resistance tests, viral subtype, CD4 count, VL at failure and previous history of VF.*
2. *Primary definition.*
3. *Defining VF as at least 2 consecutive measures >500 instead of a single value, after at least 6 months on ART.*
4. *Defining VF as a single value above the detection limit of the test used after at least 6 months on ART.*
5. *Defining VF as a single VL >1000 (instead of >500) after at least 6 months on ART.*

Table 3.10. Region and Calendar Year associations with the risk of detecting resistance in subgroup and exploratory analyses

		Definition A[2]		Definition E[3]		Definition F[4]	
		aOR (95% CI)	**Global P**	**aOR (95% CI)**	**Global P**	**aOR (95% CI)**	**Global P**
Region	*South*	*1.00*	<.0001	*1.00*	<0.0001	*1.00*	<0.0001
	Central West	0.81 (0.58 - 1.13)		0.99 (0.69-1.43)		0.95 (0.53 - 1.73)	
	North	0.29 (0.21 - 0.39)		0.33 (0.23-0.48)		0.28 (0.16 - 0.48)	
	Central East	0.47 (0.29 - 0.76)		0.50 (0.30-0.87)		1.06 (0.34 - 3.27)	
	East	1.03 (0.46 - 2.32)		1.96 (0.75-5.11)		0.68 (0.14 - 3.29)	
Calendar Year	*97-98*	0.31 (0.19 - 0.49)	<.0001	0.38 (0.22-0.66)	0.0002	0.24 (0.10 - 0.59)	0.0045
	99-00	0.47 (0.34 - 0.65)		0.43 (0.29-0.63)		0.31 (0.17 - 0.54)	
	01-02	0.75 (0.57 - 0.99)		0.64 (0.47-0.88)		0.70 (0.40 - 1.21)	
	03-04	*1.00*		*1.00*		*1.00*	
	05-06	0.73 (0.55 - 0.98)		0.67 (0.49-0.92)		0.54 (0.30 - 1.00)	
	07-08	0.90 (0.60 - 1.35)		0.77 (0.50-1.19)		0.81 (0.32 - 2.08)	
	09-10	0.49 (0.30 - 0.79)		0.43 (0.26-0.71)		0.86 (0.26 - 2.85)	
	11-12	0.68 (0.36 - 1.27)		0.47 (0.23-0.98)		0.790.27 - 2.32)	

1. *Adjusted for age, gender, ethnicity, mode of HIV transmission, previous use of mono/dual therapy, number of available previous resistance tests, viral subtype, CD4 count, VL at failure and previous history of VF.*
2. *Primary definition.*
3. *Defining failure as a single viral load >500, requiring an individual to supress to <500 before a subsequent failure was included in the analysis.*
4. *Defining VF as a single viral load >500 followed by a switch to a drug belonging to a class that was not included in the regimen started >6 months earlier*

3.5. Discussion

3.5.1. Resistance testing

The analyses in this chapter describes the changing utilisation of resistance testing in Europe, as well as the prevalence of drug resistance over time among those who had a resistance test following virological failure. The first main finding is that among all individuals experiencing VF, only around 1/3 of individuals received a resistance test within 12 months of experiencing VF, and this proportion decreased after 2004. The relatively low proportion of individuals receiving a resistance test around the date of VF extend previous EuroSIDA findings (483) to more recent calendar years. Presuming that EuroSIDA accurately captures resistance data, this indicates that there is a possible discrepancy between clinical practice and current guidelines which recommend always testing for resistance after virological failure.

Clinical decisions are complicated by numerous factors and it is not uncommon that guidelines are not followed exactly in real life. My findings are in agreement with those of Pillay et al. in the UK, who found that around 1/3 of treatment switches were guided by a resistance test. However, their analysis was carried out in a much earlier time-period (1999-2002) (479). In addition, an analysis from the US by Palella et al. from the HOPS cohort also found that around 1/3 of patients had a resistance test following VF over the study period 1999-2005 (482). In contrast, the proportion of individuals receiving a resistance test is higher in other analyses of US data (484) as well as in analyses of Canadian (481,485) and Swedish (478) data. An analysis of data from the UK CHIC cohort has shown that 46% of individuals had a resistance test after viral rebound prior to a change in therapy, similar to the estimate of 47% I found when defining failure as a high VL which was followed by a switch in the regimen (510). The higher frequency found in this subgroup suggests that one of the reasons that clinicians do not prescribe a resistance test is perceived non-adherence, which reduces the utility of resistance testing. It could be that when seen in a clinical setting, the reason for the VF may be put down to poor adherence, a chaotic lifestyle, or personal issues rather than drug resistance. This is in agreement with the higher proportion of presumed adherent individuals having a resistance test observed in the sensitivity analyses, as well as the fact that individuals of a non-white ethnicity and those who inject drugs - both groups with known adherence issues and issues regarding access to treatment (511,512) - were less likely to receive a resistance test. However, it should be noted that the validated adherence data that is not available in EuroSIDA.

In terms of time-trends, Eyawo et al., in a 2011 study of resistance testing in a Canadian cohort study, found that individuals initiating HAART after 2004 were less likely to receive a resistance

test (485); whereas Buchacz et al. found that the probability of receiving a resistance test in the US was reasonably stable over time in the period 1999-2006 (484). One North American (481) and one Canadian (475) study reported an increase in the proportion of individuals receiving a resistance test following VF over time. There are a number of additional possible reasons for these discrepancies, including differing definitions of virological failure used in the various analyses, differences in access to GRT technology in different geographical regions as well as differences in clinical practice. It is possible that a greater availability of different drug classes, as well as a changing composition of the cohort (e.g., individuals with complex treatment histories are seen more rarely in recent calendar years (492)) make clinicians less likely to test for resistance following VF. In addition, the marked reduction in the proportion of individuals experiencing VF documented here confirms previous observations that VF is growing very rare (513).

I found marked regional differences in the utilisation of resistance testing. Clinicians from Central Western and Northern Europe were more likely to offer a resistance test compared to clinicians in Southern Europe, whereas clinicians in Eastern Europe were less likely to test for resistance compared to Southern Europe. This could reflect geographical differences in healthcare expenditure as well as access to resistance testing technologies and the ability to provide this data to EuroSIDA. Individuals with a history of mono and dual therapy before starting cART were more likely to receive a resistance test in univariable analyses, but not after adjustment for previous virological failure. Although these two factors were correlated, it could suggest that the overall extent of treatment exposure due to viral failure was the factor that influenced the decision of whether to order a GRT or not, regardless of whether the person had specifically been exposed to mono or dual therapy before cART. Similarly, having a previous resistance test was also associated with an increased probability of another resistance test, as has been previously reported (483). I also found that individuals who had a low RNA at failure (<1000 copies/mL) were less likely to receive a resistance test. As mentioned previously, one of the possible explanations for this finding is technical and related to the difficulty in genotyping at low viral loads.

3.5.2. Resistance prevalence

Despite the observed declining trend in resistance testing, drug resistance was detected in a relatively high proportion, almost 80%, of tests included in this analysis. This is broadly comparable to what has been found in other studies using the same denominator to study resistance testing, where estimates ranged from 59-88% depending on the study (Table 3.2). The fact that drug resistance was detected so frequently may indicate that clinicians may be taking a selective approach to resistance testing, where those individuals judged as most likely

to have resistance are also the ones who are offered a test. In other words, where clinicians suspect poor adherence they may not order resistance tests, as performing a GRT on an individual who is not taking cART will not yield any results.

The proportion of tests detecting any resistance peaked in 2003-2004, and there was some evidence to suggest that this proportion had declined in more recent calendar years. A decline in the prevalence of resistance in recent years among individuals experiencing VF has been found in a number of other studies in high income settings (336,478,486,488–491,514), as outlined in the literature review (Table 3.2). Such a decrease could possibly be explained by improvements in the potency of drugs used, minimized side effects and an increase in therapeutic options that have all lowered the risk of developing drug resistance (336,514). This could have led, in turn, to lower levels of transmitted resistance and consequently even fewer people failing because of resistance when starting these potent new cART regimens from an ART-naïve status. Simplified drug regimens that are easier to take, combined with efforts to educate HIV positive people, and the development and use of drugs with a high genetic barrier may also play an important role. Taken together with the reduced proportion of individuals experiencing VF in more recent calendar years, the current analysis documents a marked reduction in the number of individuals experiencing VF with drug resistance, echoing findings from other European cohorts (515).

An other predictor of detecting resistance was geographical region. Conditional on having a GRT done, the probability of detecting resistance when doing a test was lower in Northern and Central Eastern Europe compared to Southern Europe. I also found that individuals who had a history of mono or dual therapy were more likely to have detected resistance, as were individuals who had experienced previous virological failure, in agreement with a previous analysis of US data by Napravnik et al (498). We found some evidence that people who inject drugs were less likely to have detected resistance compared to MSM, as mentioned previously this could be explained by the fact that PWID are typically less adherent to treatment than MSM.

Overall, these results indicate that clinical attitudes to resistance testing may differ according to geographical region. Clinicians in Northern Europe were the most likely to prescribe a resistance test following VF, but also least likely to see drug resistance in the virology report once the test was performed. In contrast, clinicians in Eastern Europe were comparatively less likely to test for resistance following VF compared to Central Western and Northern Europe, but also more likely to observe resistance when a test was done compared to Central Eastern and Northern Europe. As it is unlikely that the biological risk of developing drug resistance in a situation of VF varies by region of Europe, it is possible that the lower testing rates in Eastern Europe are causing

individuals to be maintained on failing therapies for longer, thus leading to the development of resistance. Clinicians in Eastern Europe may also be even more selective about who they test for resistance, targeting those who they are certain have failed due to resistance. This has also been indicated in a recent survey of the EuroSIDA clinics (431).

By using the proportion with a GRT in the denominator in this analysis, I have been able to describe the likelihood of observing resistance when ordering a test from a clinical point of view. Although this helps us understand the situation and potential clinical strategies chosen by clinicians in different regions of Europe, or in different calendar years, it is important to note that the prevalence estimates here do not represent population-level prevalence estimates of the burden of resistance. Such an estimate would require either assuming that all individuals without a GRT did not harbour resistance, or using some form of multiple imputation. This latter approach has already been taken in a previous EuroSIDA publication by Bannister et al. (473).

3.5.3. Limitations and strengths

The analyses presented here are subject to several limitations. First of all, my chosen definition of VF may not capture all VFs, particularly as the definition for this has changed over time. I chose the definition used by the European AIDS Clinical Society treatment guidelines at the time of conducting this analysis, as this is likely to be broadly representative for clinics across Europe. It was also a relatively non-strict definition of VF, which allowed me to maximise the sample size. However, it is likely that what a clinician considers to be VF has varied over time and is different across different geographical regions. I attempted to address this possible bias in sensitivity analyses, and although the proportion receiving a test was higher using more stringent definitions of VF, it still remained relatively low and decreased over time. However, I cannot exclude that some instances of VF have been misclassified. If the definition I used for VF is too relaxed, this means that I have classified individuals as experiencing VF even though they're not, which could lead to an underestimate of the proportion tested. However, if the definition used for VF is too stringent, for example in the most recent calendar years, this could lead to an overestimate of individuals who are tested. The way this bias operates is likely to vary over time, making it complex to predict what the effects on my results are.

Another potential limitation is that the number of resistance tests in the EuroSIDA database may not accurately reflect all the tests that are done as part of routine clinical care. Although rigorous efforts are made to minimise such under-reporting by quality control visits as outlined in Chapter 2, Section 2.2.1.2, the financial incentive for clinicians to return complete resistance forms as compared to other aspects of the EuroSIDA CRF is relatively low. It is possible that the lower rate of resistance testing found in Eastern Europe simply reflects the fact that less data is

reported from these clinics. However, my results are in agreement with an additional survey of self-reported clinical HIV management conducted in EuroSIDA centres, which should be less affected by a lack of financial incentives (431). The number of tests done in recent calendar years may also have been underestimated due to reporting delay.

As with all observational studies, I cannot rule out the possibility that there are unmeasured confounding variables affecting some of the associations reported here. Although I was able to evaluate a large number of clinically relevant predictors of receiving a resistance test and detecting predicted resistance, there may be other factors, such as the availability of different drugs, that I was not able to assess in multivariable models that may explain some of the observed associations. Finally, EuroSIDA clinics may not be representative of all HIV clinics or the entire population of HIV infected individuals in Europe, as some countries are represented by relatively few centres of excellence. Therefore, the results may not be generalizable to the HIV-infected population in Europe as a whole.

Despite these limitations, the analysis does have a number of strengths. EuroSIDA is one of few studies that collect data from centres across the European region, including from countries in Eastern Europe. Although the estimates from the Central Eastern and Eastern European region presented here may suffer somewhat from limited generalisability, there is scarce data on resistance testing and prevalence from the region as a whole. As the EuroSIDA cohort has been running since 1994, I was able to assess time-trends for a considerable time-period. The breadth of variables collected also allowed me to evaluate a reasonably large number of potential risk factors, which most surveillance databases cannot do.

3.5.4. Conclusions

To conclude, my findings indicate that the clinical approach to resistance testing may diverge from that laid out in guidelines, and I observed calendar year and regional differences both in resistance testing and the probability of detecting resistance. Public health policy aiming at minimizing the emergence of drug resistance might benefit from targeting specific regions of Europe, and efforts to minimize inter-regional differences in the availability and utilisation of resistance testing in the European Union may be warranted. The implications of these findings for further research and policy will be discussed further discussed in Chapter 8 of this thesis.

3.5.5. Dissemination of results

Preliminary results from this chapter were presented as an oral presentation at the 2013 European AIDS conference and were published in AIDS in 2015 (Appendix VI).

Chapter 4 . Long-term virological outcomes and resistance patterns among treatment experienced HIV patients receiving raltegravir

4.1.Introduction and Objectives

Traditionally, HIV has been treated with a combination of drugs from three main drug classes: nucleoside reverse transcriptase inhibitors (NRTI), non-nucleoside reverse transcriptase inhibitors (NNRTI) and protease inhibitors (PI). However, as outlined in Chapter 1, drugs targeting other stages of the viral life cycle have also been developed. The recently licensed integrase strand transfer inhibitors (INSTI) prevents the HIV genome from replicating by inhibiting the process of viral integration (516). Viral integration consists of two main steps: 3' processing and strand transfer. 3' processing involves the removal of a dinucleotide from the end of the viral DNA. This cleaved DNA product is consequently covalently linked to the host genome through a process called strand transfer (517). INSTIs attach to the integrase by binding to metal cations (Mg2+ or Mn2+) present in the active site. This prevents the integrase from binding to the viral DNA, and thus inhibits the strand transfer step of integration (518). The first developed INSTI, raltegravir (RAL; Isentress® Merck & Co), has been licensed for use in Europe since 2007, its structural analogue elvitegravir (ELV) was licensed in 2013 and the second generation INSTI dolutegravir (DTG) was approved in 2014. These drugs have grown more popular over time due to their high virological efficacy and favourable side-effect profiles, and INSTIs are now recommended as an alternative option for use as part of first line therapy in many high income settings (146,147).

RAL is the most well-studied among the INSTIs, and has been shown to have high virological efficacy among both treatment naïve (519–521) and experienced (522–524) patients. Compared to other first-line third agent options, such as the NNRTI efavirenz (EFV), RAL appears to be associated with fewer central nervous system (CNS) related side-effects (525). Interestingly, data from the STARTMRK trial has suggested that patients receiving RAL may experience a faster VL decrease compared to patients receiving EFV (519). However, RAL also has a low genetic barrier to resistance, and a single mutation is sufficient to confer high level resistance to RAL (526–528).

Individuals who fail RAL have been found to develop resistance according to 3 main pathways involving positions Y143, N155 or Q148 (527), usually in combination with characteristic secondary mutations (Table 4.1) (528).

Table 4.1 Primary resistance pathways for RAL

Resistance Pathways	RAL fold resistance, relative to wild-type (WT)
Y143 pathway	
Y143C	<10
Y143R	<50
Y143C/T97A	>100
Y143R/T97A	>100
Y143G/T97A/L74M	<50
Y143G/T97A/L74M/E138A	<20
N155 pathway	
N155H	<50
N155H/E92Q	<100
N155H/L74M	<50
Q148 pathway	
Q148H	<20
Q148K	<100
Q148R	<50
Q148H/E138K	<10
Q148K/E138K	>100
Q148R/E138K	>100
Q148H/G140S	>100
Q148K/ G140S	<10
Q148R/ G140S	>100
Q148H/E138A/G140S/Y143H	>100

Adapted from Quashie et al, ref: (529)

Both the Q148 and Y143 mutations adversely affects the function of the integrase, but this is compensated for by the emergence of the G140S and T97A mutations (530). Although the emergence of substitutions in position N155 also has negative effects on viral fitness, the E92Q mutation that characteristically follows N155H does not compensate this loss to the same extent as G140S or T97A. This means that N155H pathways tend to be replaced over time in the presence of selective drug pressure (528,529).

Mutations in position N155 and Q148 confer resistance to ELV in addition to RAL, which means that RAL and ELV cannot be used sequentially (528). However, DTG has a higher barrier to resistance and there is less cross-resistance between RAL/ELV and DTG. The VIKING-III trial showed that patients who have previously failed RAL can re-supress on DTG (531). However, it was found that they are less likely to do so if they carry the Q148 resistance mutation (527), particularly if this is present in combination with mutations at position 138, 140, 92 and 97 (528).

Data from clinical trials indicate that rates of virological failure with resistance are likely to be low for RAL, although they do vary according to treatment history. Estimates range from 0-3% among patients starting RAL from naïve, 2-7% among those starting RAL from treatment experienced and up to 20% amongst individuals starting RAL following triple class failure (TCF) (Table 4.2). However, clinical trials often have very strict inclusion criteria coupled with careful and regular monitoring of patients, conditions which may not be replicated in routine care. Adherence to medication may also be higher in clinical trials than in clinical practice (532). As RAL requires twice-daily dosing and high adherence to prevent resistance from emerging (533,534), studying its long-term efficacy and risk of resistance development in routine practice is of informative, and can complement the existing information from trials.

The objectives of this chapter were therefore to:

1) Describe time to failure and risk factors for failing RAL
2) Describe resistance patterns among those failing RAL with available resistance test results

Table 4.2. Phase III trials evaluating RAL efficacy or non-inferiority, virological outcomes and INSTI resistance prevalence

Trial	Year	Comparison	Population[1]	Virological Outcome	INSTI Resistance (%) [2]	Reference
NEAT 001/ANRS 143 (96 weeks)	2016	DRV/r+RAL OR TDF/FTC	TN	19% Treatment Failure	14 (5/36)	(535)
ACTG 5257 (96 weeks)	2014	FTC/TDC + ATV OR RAL OR DRV	TN	94% VL<50	17 (11/65)	(292)
SPRING-2 (96 weeks)	2014	OBT + RAL OR DTG	TN	76% VL<50	5 (1/20)	(232)
SPRING-2 (48 weeks)	2013	OBT + RAL OR DTG	TN	85% VL<50	5 (1/19)	(536)
STARTMRK (240 weeks)	2013	FTC/TFD + RAL OR EFV	TN	71% VL<50	17 (4/23)	(519)
PROGRESS (96 weeks)	2013	LPV/r + RAL or TDF/FTC	TN	66.3% VL<40	37.5% (3/8)	(537)
SPARTAN (24 weeks)	2012	ATV + RAL OR TDF/FTC	TN	74.6% VL<50	66 (4/6)	(538)
PROGRESS (48 weeks)	2-11	LPV/r + RAL or TDF/FTC	TN	83.2% V<40	25 (1/4)	(539)
STARTMRK (156 weeks)	2011	FTC/TFD + RAL OR EFV	TN	75.4% VL<50	44 (4/19)	(520)
STARTMRK (96 weeks)	2010	FTC/TFD + RAL OR EFV	TN	81% VL<50	33 (4/12)	(521)
STARTMRK (48 weeks)	2009	FTC/TFD + RAL OR EFV	TN	86.1% VL<50	44 (4/9)	(228)
SECOND-LINE (96 weeks)	2015	LOP/r+2 /3NRTI OR RAL	TE	80% VL<200	Not reported	(540)
SAILING (48 weeks)	2013	OBT + RAL OR DTG	TE	64% VL<50	42 (16/38)	(233)
Study 145 (96 weeks)	2013	PI/r+1 additional drug + RAL OR EVG	TE	45% VL<50	28 (26/93)	(541)
SECOND-LINE (48 weeks)	2013	LOP/r+2 /3NRTI OR RAL	TE	83% VL<200	14 (7/49)	(542)
Study 145 (48 weeks)	2012	PI/r+1 additional drug + RAL OR EVG	TE	58% VL<50	22 (15/72)	(230)

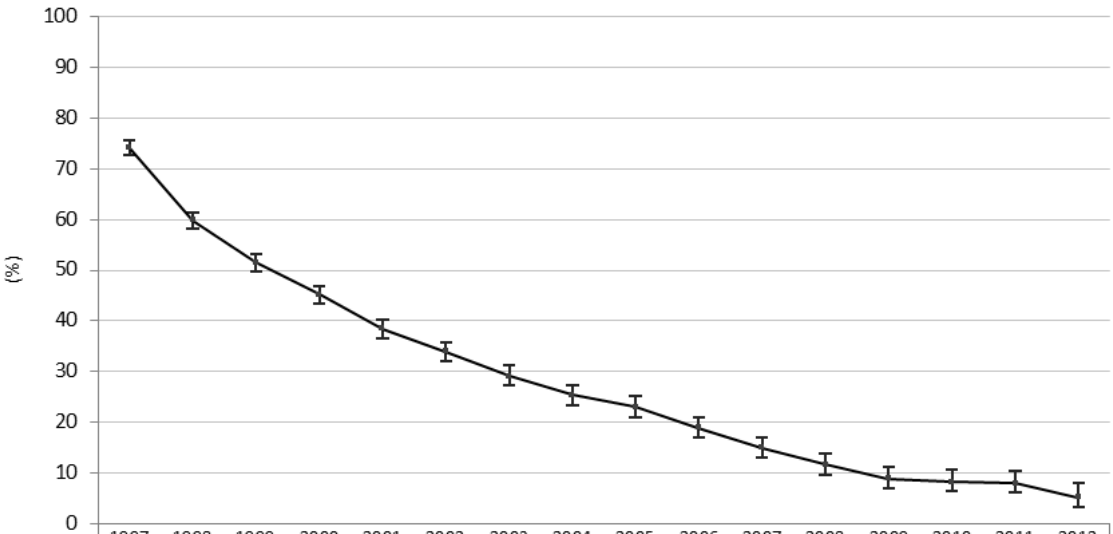
SWITCHMRK (24 weeks)	2010	OBT + LOP/r or RAL	TE	84% VL<50	57 (8/14)	(229)
BENCHMRK (240 weeks)	2013	OBT + RAL OR Placebo (RAL offered after week 156)	TCF	51% VL<50	64 (95/148)	(524)
BENCHMRK (96 weeks)	2010	OBT + RAL OR Placebo	TCF	57% VL <50	Not reported	(523)
BENCHMRK (48 weeks)	2008	OBT + RAL OR Placebo	TCF	62.1% VL<50	Not reported	(522)

1. TN=Treatment Naïve; TE=Treatment Experienced; TCF=Triple Class Failure, OBT=Optimized Background Therapy
2. Prevalence of INSTI resistance reported as a % of those successfully genotyped, raw numbers given within brackets (numerator/denominator).

4.1.1.Trends in virological failure and baseline characteristics

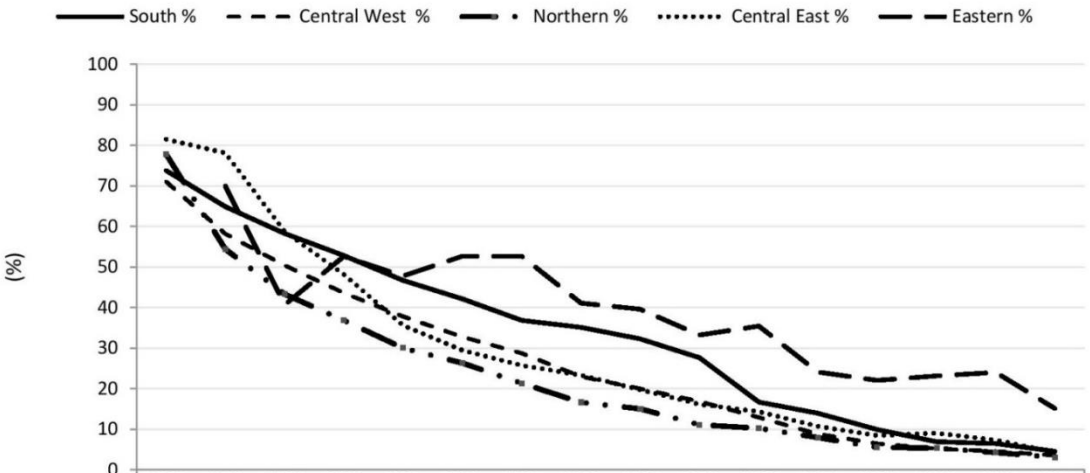
8,469 individuals out of 18,473 in D36 (46%) experienced VF (as defined by a VL > 500 copies/mL) at least once and were included in the analysis for Aim 1 (Figure 3.2). The probability of experiencing VF declined over time: compared to 74.2% (95%CI=72.9-75.4) of individuals with at least one VL measurement in 1997, only 5.1% (95%CI=3.2-8.0) showed evidence of VF in 2012 (Figure 4.2). As can be seen in Figure 4.1, the decline appeared to be nearly linear, and it was therefore decided to model calendar year as a linear variable when identifying risk factors for VF. This decline in the probability of VF was consequently confirmed in multivariable models (Adjusted Odds Ratio [aOR]=0.79 per more recent calendar year, 95% Confidence Interval [95%CI]= 0.78-0.79, p<.001). Including an interaction term in the model showed that there was evidence that this decline differed according to geographical region (interaction p-value<0.001), with the odds of experiencing VF declining most steeply in Central Eastern Europe (aOR=0.90, 95%CI=0.87-0.93, p<0.001 and less markedly in Eastern Europe (aOR=0.95, 95%CI=0.91-0.99, p=0.01). In the most recent calendar year for which we had data (2012) the lowest absolute levels of virological failure were seen in Northern Europe (3.0%) and the highest levels in Eastern Europe (15.1%; Figure 4.2).

Figure 4.1. Proportion of individual on ART each year who experienced VF with a VL>500 copies/mL



	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012
Total	4654	6090	6352	6478	6720	6776	6876	7182	7373	7527	7845	8028	8285	7832	7892	6596
Failing N	3453	3648	3270	2924	2583	2296	2000	1818	1693	1422	1168	927	730	642	622	337
Failing %	74.19	59.9	51.48	45.14	38.44	33.88	29.09	25.31	22.96	18.89	14.89	11.55	8.81	8.2	7.88	5.11

Figure 4.2. Proportion of individuals on ART each year who experienced VF with a VL>500 copies/mL, by region



	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012
South %	73.8	64.9	58.3	52.8	46.7	42.1	36.8	35.1	32.3	27.6	16.7	13.9	9.9	6.9	6.4	4.5
Central West %	71	58.2	50.4	43.6	37.8	32.7	28.7	23	20	16.8	12.9	8.8	6.4	5.2	4.5	3.5
Northern %	77.8	54.4	43.3	36.8	30	26.3	21.3	16.6	15	11.1	10.2	7.9	5.5	5.3	4.2	3
Central East %	81.5	78.1	58.7	48.2	35.6	29.4	25.7	23.3	19.7	16.1	14.3	10.7	8.4	9	7.3	4.3
Eastern %		70	40.7	52.6	47.8	52.7	52.7	41.1	39.6	33.2	35.4	24.1	22	23.1	24	15.1

The characteristics of the individuals showing evidence of VF at date of the first VF are shown in Table 4.3. The majority of individuals included were male (75%) and white (86%), and 40% had acquired their HIV infection through sex with another man. There was reasonable representation from all geographical regions, with the largest number of participants coming from Southern Europe (2639, 31%), followed by Central (2175, 26%) and Northern (2090, 25%) Europe. 743 participants came from Central Eastern Europe (9%) and 607 (7%) from Eastern Europe. In terms of treatment history, the majority of the individuals had been exposed to mono/dual therapy (68%), although fewer had met our pre-specified definition of virological failure before their baseline date (21%) and very few had received a resistance test (4%). In terms of current ART regimens, the majority of the participants received cART - defined as receiving at least 3 drugs - at baseline (85%). The most common regimen among these included unboosted PI's (35.2%), followed by boosted PIs (18.4%). This possibly reflects the relatively early calendar year most participants were included, with a median baseline date of October 1997 (IQR=March 1997-September 2000). Laboratory markers indicated that individuals were doing relatively poorly, with a median CD4 count of 289 (167-446) and a median viral load of 4233 (1200-24,000).

The median VL at the first failure varied with calendar year, and decreased somewhat from 5831 (1500-30000) copies/mL in 1997/1998 to 4378 (1230-21902) copies/mL in 2011/2012 ($p<0.001$). Conversely, the number of VL measurements per patient per year decreased over time, from a median of 4 (3-6)/year in 1997/1998 to 3 (2-4)/year in 2011/2012 ($p<0.001$).

Table 4.3. Baseline Characteristics of the Study Population

		Total experiencing VF	Did not have resistance test	Had resistance test[1]	
		N (%)	N (%)	N (%)	P-value[2]
Demographics		8469 (100)	5793 (100)	2676 (100)	0.006
Gender	*Male*	6344 (74.9)	4288 (74.0)	2056 (76.8)	0.03
	Female	2125 (25.1)	1505 (26.0)	620 (23.2)	
Ethnicity	*White*	7305 (86.3)	5030 (86.8)	2275 (85.0)	
	Non-white	1164 (13.7)	763 (13.2)	401 (15.0)	
Risk Group	*MSM*	3401 (40.2)	2212 (38.2)	1189 (44.4)	<.001
	IDU	2068 (24.4)	1530 (26.4)	538 (20.1)	
	Heterosexual	2381 (28.1)	1640 (28.3)	741 (27.7)	
	Other	619 (7.3)	411 (7.1)	208 (7.8)	
Region	*Southern*	2639 (31.2)	1856 (32.0)	783 (29.3)	<.001
	Central	2175 (25.7)	1382 (23.9)	793 (29.6)	
	Northern	2090 (24.7)	1296 (22.4)	794 (29.7)	
	Central East	743 (8.8)	560 (9.7)	183 (6.8)	
	East	607 (7.2)	541 (9.3)	66 (2.5)	
Treatment History					
Type of ART used at failure	*PI (boosted)*	1560 (18.4)	979 (16.9)	581 (21.7)	<0.001
	PI (unboosted)	2981 (35.2)	2030 (35.0)	951 (35.5)	
	NNRTI	812 (9.6)	635 (11.0)	177 (6.6)	
	PI and NNRTI	1431 (16.9)	965 (16.7)	466 (17.4)	
	Neither PI or NNRTI	1685 (19.9)	1184 (20.4)	501 (18.7)	

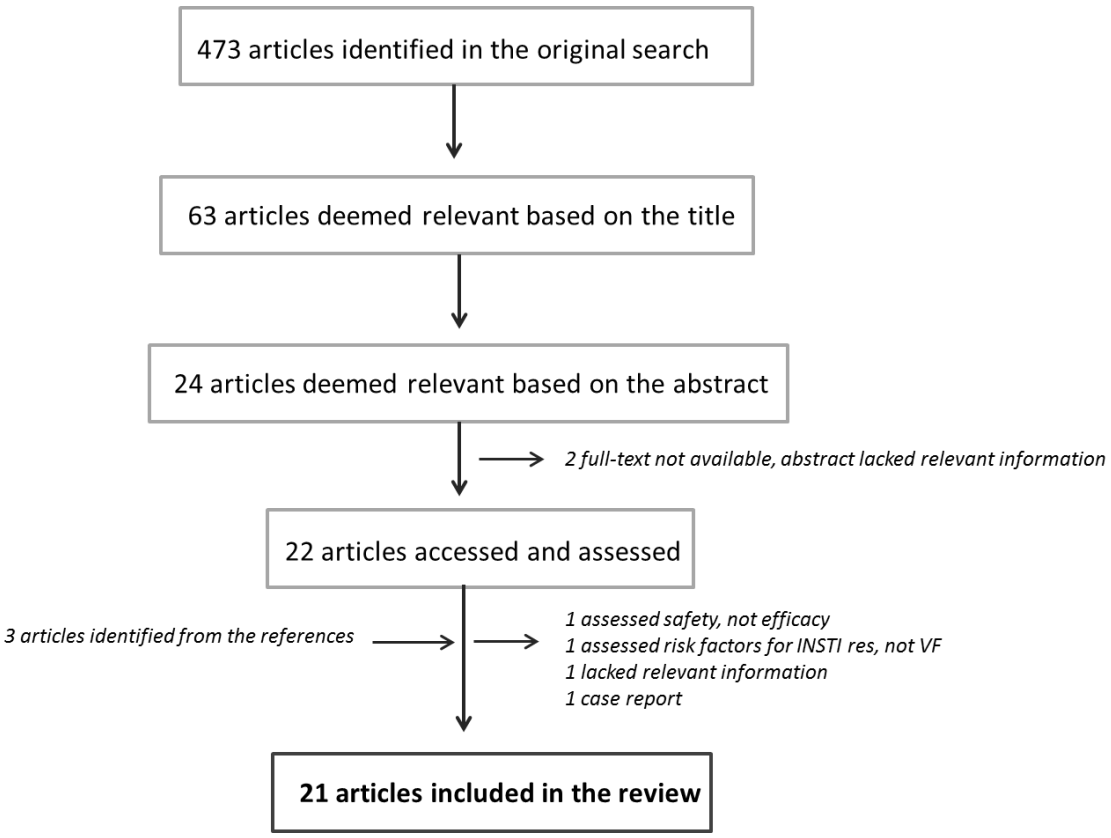
History of mono/dual therapy	*No*	2688 (31.7)	2080 (35.9)	608 (22.7)	<0.001
	Yes	5781 (68.3)	3713 (64.1)	2068 (77.3)	
On CART	*No*	1284 (15.2)	889 (15.3)	395 (14.76)	0.49
	Yes	7185 (84.8)	4904 (84.7)	2281 (85.2)	
Previous VF	*No*	6666 (78.7)	4721 (81.5)	1945 (72.7)	<0.001
	Yes	1803 (21.3)	1072 (18.5)	731 (27.3)	
Previous resistance test	*No*	8121 (95.9)	5560 (96.0)	2561 (95.7)	0.55
	Yes	348 (4.1)	233 (4.0)	115 (4.3)	
Continuous variables		**Median (IQR)**	**Median (IQR)**	**Median (IQR)**	
Age	*Year*	38 (33.0 - 44.7)	38 (33.0 - 44.9)	38 (33.0 - 44.3)	0.44
CD4	*Cells/mm3*	289 (167.0 - 446.0)	299 (171.0 - 459.0)	271 (157.0 - 420.0)	<.001
RNA	*Copies/mL*	4233 (1200 - 24000)	3599 (1080 - 21200)	6072 (1600 - 29000)	<.001

1. *Defined as in section 3.3.1; requiring either the presence of resistance results in the EuroSIDA database or an indication by the clinician that a test was done.*
2. *P-values compare those with and without a resistance test, and are calculated using a chi-squared test (categorical variables) and Kruskal-Wallis (continuous variables).*

4.2. Literature Review

I searched the literature for observational studies reporting virological outcomes and/or integrase resistance prevalence among individuals receiving RAL in routine clinical care. The search terms are specified in Chapter 2. The original search returned 474 articles, of which 63 were deemed potentially relevant. Following abstract screening, 24 records were deemed relevant. Two of these did not have full-text versions available, and the abstract lacked key information. Therefore, 22 articles were accessed and assessed for inclusion in the review. In total, 21 articles were included, and the review process is outlined in Figure 4.3.

Figure 4.3. Selection process for the literature review



The results from the literature review are presented in Table 4.4. A large number of different definitions were used to define virological outcomes. The majority of the studies, 17/21, used an estimate of virological suppression (VS) as the endpoint whereas 4 studied virological failure (VF). There was also large variation in the cut-off points, with 40, 50 and 200 copies/ml all used to indicate VS/VF. For studies where more than one cut-off point was used, I chose to summarise the results using only the highest VL cut-off. Where the probability of VS/VF was assessed using Kaplan Meier methods and reported at a certain time-point, this time-point is specified in the table. Estimates were commonly reported separately according to whether patients started RAL with a supressed viral load (SVL) versus a raised viral load (RVL). Different methods were also used to analyse time to event data, with both Intention to Treat (ITT) and Per Protocol (PP) approaches reported. Finally, different methods for dealing with missing data were reported. Although none of the studies used multiple imputation, there were some differences in estimates according to whether individuals with missing viral loads were classed as failures ("m=f") or whether missing data was reduced using a more liberal last-observation-carried forward (LOCF) approach.

Table 4.4. Observational studies reporting virological efficacy data for RAL and/or INSTI resistance prevalence

Author	Year	Population[1]	N (FU time) [2]	Virological Outcome[3]	Risk Factors for outcome[4]	INSTI Resistance (%) [5]	Ref[6]
Santos	2015	TE/TN	2,782 (*NR*)	4.4% VL>200	Higher VL and low adherence.	79 (26/33)	(543)
Jaeckle	2015	TE/TN	295 (24/48 weeks)	71% VL<200, 24 weeks 69% VL<200, 48 weeks	*NR*	52 (12/23)	(544)
Van Halsema	2015	TE/TN	215 (*NR)*	4.70%. *NR*	*NR*	50 (2/4)	(545)
Squires	2013	TE/TN	206 (48 weeks)	64% VL<50, RVL 76% VL<50, SVL	*NR*	29 (11/38)	(546)
Gras	2012	TE/TN	81 (6 months)	15% VL>40	Adherence and duration of treatment interruption but not raltegravir plasma levels or prior duration of viral suppression	20 (2/10)	(534)
Buchacz	2015	TE	472 (1 year)	68% VL<50	*NR*	*NR*	(547)
Fourati	2014	TE	502 (*NR)*	*NR*	*NR*	39 (194/502[7])	(548)
Capetti	2014	TE	333 (4 years)	73% VL<50, ITT 93.4% VL<50, PP	Risk of VF higher with higher baseline VL and having a complex treatment history.	58 (15/26)	(549)
Marcelin	2013	TE	468 (6 months)	71%% VL<50	Lower baseline VL, higher baseline CD4 count, use of two or more new drugs among DRV/ETR/MVC or ENF	78 (49/63)	(550)
Capetti	2012	TE	320 (96 weeks)	8.4% VL>50, ITT *NR*, PP	Lower CD4 counts, Advanced AIDS (ITT) None (PP)	79 (11/14)	(551)

Armenia	2012	TE	23 (24 weeks)	39% VL<50	None	57 (8/11)	(552)
Da Silva	2010	TE	51 (6 months)	78% VL<50	*NR*	81 (9/11)	(553)
Engsig	2010	TE	32 (72 weeks)	100% VL<51	*N/A*	*N/A*	(554)
Towner	2009	TE	52 (24 weeks)	94.20% VL<50	*NR*	*NR*	(555)
Wittkop	2009	TE	51 (24 weeks)	78% VL<1.7 $\log_{10}$ copies	Baseline VL and nadir CD4	81 (9/11)	(556)
Grant	2009	TE	14 (24 weeks)	86% VL<50	*NR*	*NR*	(557)
Rusconi	2013	TCF	105 (24 weeks)	74.3% VL<50	Older age, higher GSS and time in cohort	50 (12/24)	(558)
Bucciardini	2012	TCF	101 (48 weeks)	25.7% VL>50	None identified	*NR*	(559)
Scherrer	2010	TCF	243 (24 weeks)	69.2% VL<50, m=f, RVL 80.9% VL<50, m=f, SVL 76.1% VL<50, LOCF, RVL 95.8% VL<50, LOCF, SVL	None	*NR*	(560)
Teague	2010	TCF	57 (48 weeks)	84% VL<50, RVL 92% VL<50, SVL	*NR*	*NR*	(561)
Harris	2008	TCF	35 (7 months, median)	97% VL<50	*NR*	*NR*	(562)

1. N=Treatment Naïve; TE=Treatment Experienced; TCF=Triple Class Failure.
2. NR=Not Reported
3. ITT=Intention to Treat; PP=Per Protocol; m=f = missing equals failure; LOCF= Last observation carried forward
4. Can be either risk factors for VF or factors associated with suppression; the outcome is specified in the "Virological Outcome" column.
5. Prevalence of INSTI resistance reported as a % of those successfully genotyped, raw numbers given within brackets (numerator/denominator).
6. Ref=Reference
7. The proportion with VF; the proportion genotyped not reported

4.2.1. Virological outcomes

Although a reasonably large number of observational studies were identified, none of them assessed virological outcomes among treatment naïve patients alone, and the majority of the studies only included treatment-experienced patients. Five studies studied efficacy exclusively among patients who had experienced TCF. Although it is hard to compare results across studies given the large number of different definitions used for VS/VF, the variable duration of follow-up time and the different study populations, the vast majority of studies appeared to find favourable outcomes of RAL and drew positive conclusions regarding the efficacy of RAL. Among studies including both treatment experienced and treatment naïve individuals, estimates of virological efficacy ranged from 64-76% VS and 4-15% VF (Table 4.4). Unsurprisingly, these proportions varied according to the time-point studied. Jaeckle et al analysed data from a clinical database in Germany, and described estimates of constant VS which decreased somewhat with increasing FU-time: from 71% at 24 weeks to 69% at 48 weeks (544). Such a decline was also observed in clinical trials (522–524).

Squires et al described results from the industry-sponsored REALMRK cohort of patients in clinical centres in the US, Brazil, South Africa and Jamaica (546). The aim of the REALMRK study was to describe the efficacy of RAL in a more diverse setting than that studied in clinical trials. The study recruited a higher proportion of women and individuals of non-white ethnicities compared to the BENCHMRK studies, and followed individuals for 48 weeks. REALMRK reported good efficacy of RAL, with 76% of patients who switched to RAL or started the drug from ART- naïve reaching virological suppression by 48 weeks. Rates were lower when RAL was used as salvage therapy: 54% (546).

Results were more diverse in the studies included in the review conducted on treatment experienced patients, where estimates of virological suppression ranged over 39-100% according to the time frame used and the study population included (552,554). The particularly low efficacy estimate of 39% by 24 weeks comes from an analysis by Armenia et al of treatment experienced patients starting RAL at 4 clinical centres in Italy. However, only 23 patients were included in this analysis, all were heavily treatment experienced with a high viral load at baseline and a low baseline GSS. In addition, the aim of this analysis was to assess the effect of baseline polymorphisms to RAL on virological response rather than to study virological efficacy (552). In contrast, the estimate of 100% efficacy by 72 weeks comes from the Danish population based cohort – and again, reflects a very small (N=32) population (554). Smaller studies are more likely to report more extreme results than larger studies as a result of a number of biases, including publication bias (563). The studies including more than 100 individuals all reported virological efficacy around 70% in ITT analyses irrespective of the time-

frame applied (547,549,550). Per-protocol analyses did, as expected, generate a higher probability of virological suppression: 93.4% (549). Only one study estimated the risk of VF specifically. In the Italian SALIR-E cohort, 8.4% of patients experienced VF by 96 weeks in ITT analysis. Per protocol estimates of the risk of VF were not reported (551).

Estimates of virological suppression among patients with TCF also varied, and ranged from 70-97%. The largest of these studies was an analysis by Scherrer et al from the Swiss Cohort Study (560). They included patients who experienced TCF and studied virological response to RAL-based regimens at 24 weeks. The first key conclusion from their analysis is the fact that the probability of virological suppression varied depending on whether patients started RAL as salvage treatment or as a switch for safety reasons. At 24 weeks, 76.1% of salvage patients reached VS as compared to 95.8% of switch patients (560). Sherrer et al also report the impact of using different methods for handling missing data, and show that using an approach such as LOCF lead to considerably more favourable outcomes compared to using a missing=failure approach, which most trials adopt. However, due to the large proportion of missing data present in the Swiss cohort study, and indeed in many studies, the authors argue that the LOCF results are the most informative for this context (560). The difference in outcomes between salvage and switch patients was also found in an analysis by Teague et al, which described outcomes for TCF patients receiving care at the Chelsea and Westminster hospital in London. They found that 84% of salvage patients compared to 93% of switch patients achieved a supressed viral load by 48 weeks (561). Again, only one study estimated the risk of VF directly. Bucciardini et al analysed the probability of VF by 48 weeks in the Italian ISS-NIA cohort of individuals experiencing TCF who started INSTIs, and found a cumulative probability of VF at 26% (559).

In terms of predictors of virological outcomes, these were only reported in 9/21 studies. A higher baseline viral load was consistently associated with the risk of failure to achieve VS (543,550,551,556). Baseline (550,551) and nadir (556) CD4 count was also associated with virological outcomes, as was a complex previous treatment history (551,558). Marcelin et al, in a 2013 analysis of the French ANRS cohort, also found that the use of two or more new drugs among DRV/ETR/MVC or ENF together with RAL was predictive of virological success (550). Adherence was associated with worse virological outcomes in two studies (534,543). One of these, the RALTECAPS study by Gras et al, utilised a validated measure of adherence (the MEMS caps system). They found that although RAL plasma levels were not predictive of VF, adherence patterns, in particular the duration of the treatment interruption, was a strong predictor of VF (534). No demographic determinants of virological outcomes were reported, apart from older age reported as being predictive of achieving VS in one study (558).

4.2.2. Integrase resistance prevalence following RAL VF

Although the majority of the studies in the review reported on integrase resistance patterns following VF, the analyses generally involved a very small number of individuals due to the relative rarity of experiencing VF. As a proportion of those genotyped, the prevalence of integrase resistance ranged from 20% (534) to 81% (553,556). Clinical trials also report very varied prevalence of integrase resistance (Table 4.2), with estimates ranging from 5-66%. The largest observational study of integrase resistance prevalence identified for this review was undertaken by Fourati et al in the French ANRS cohort. They found a prevalence of integrase resistance of 39% among individuals experiencing VF on RAL, although the proportion of successfully genotyped samples was not reported (548). The N155 and Q148 resistance pathways predominated, followed by Y143. Although no patient was found to harbour the R263K Dolutegravir resistance mutation, 13.9% of all patients failing were still predicted to be resistant to Dolutegravir, primarily as a consequence of the Q148 mutation in combination with the G140 substitution (548). Only one study reported specifically using next generation sequencing (NGS) techniques to study minority variants (552). Armenia et al used the Ultra-Deep-454 pyrosequencing platform to study the impact of low-frequency baseline polymoprhisms on response to RAL containing regimens, and also performed ultra-deep sequencing (UDS) of 11 individuals who failed RAL. Their analysis also found 3 main resistance pathways involving either Y143, Q148 or N155 (552).

4.2.3. What this analysis adds

Virological outcomes following the initiation of RAL-based regimens have been assessed in a relatively large number of observational studies in resource-rich settings, but the majority of these have been small, restricted to a single country and only reported on shorter-term outcomes. The number of studies reporting on risk factors for RAL failure or on integrase resistance patterns is also relatively small. Only one study reported using NGS to study the minority integrase resistance mutations, which could influence the response to consequent therapy. EuroSIDA is one of few large HIV cohorts that collect data from across Europe in a standardized manner with long-term follow-up, meaning that this analysis contributes important data on RAL virological outcomes and resistance patterns in different European regions. In addition, the availability of stored samples within EuroSIDA provides an opportunity to study integrase resistance patterns despite the fact that genotyping of the integrase gene is still not commonly done in routine practice in all European countries through genotyping of samples from the plasma repository. Centrally genotyping individuals who fail RAL also allows us to apply NGS technology, which has the potential to generate important information on minority variants that would not have been possible to study using routinely collected data which is done using Sanger sequencing.

4.3. Methods

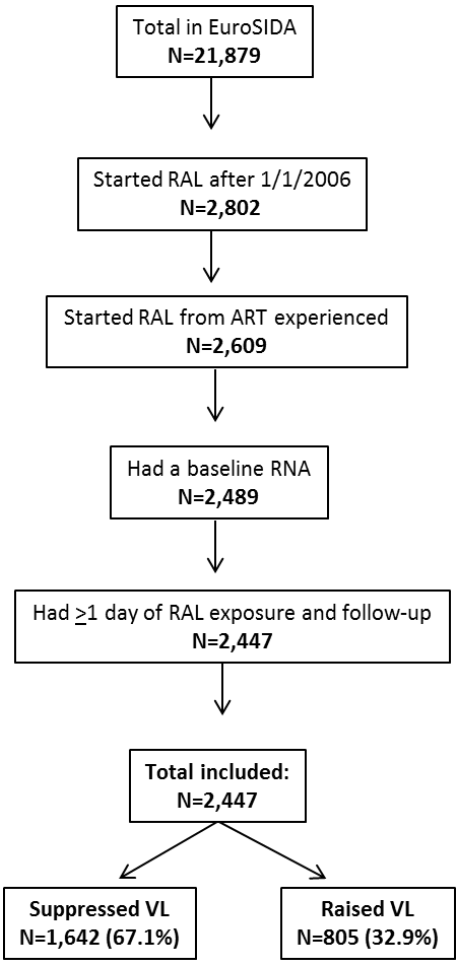
4.3.1. Inclusion criteria and study population

I used the D42 (February 2016) version of the EuroSIDA database for this analysis, which held data on 21,879 individuals. Due to the timing of the analyses, I was restricted to use resistance test results from earlier dataset rounds (June 2013). The samples selected for retrospective genotyping were selected from the March 2015 update of the repository database. The inclusion process is outlined below in Figure 4.4. Briefly; I included ART-experienced individuals who started RAL for the first time after 1/1/2006 with no prior exposure to INSTIs. The 1/1/2006 was chosen as a cut-off date as it is approximately two years prior to EMA approval of RAL, which allows for the inclusion of individuals who may have accessed RAL through compassionate early-access programs. As very few individuals (N=193, 7%) started RAL from ART-naïve it was not possible to conduct a separate analysis of this patient group, and to ease the interpretations of my findings these individuals were excluded. I further required individuals to have a baseline VL measurement and at least 1 day of follow-up with RAL exposure in order to be included. Baseline was defined as the first date of starting the RAL-based regimen. Patients were separated into two different groups:

- RAL start with a raised VL (RVL group): Started RAL with a baseline viral load (bVL) >200 copies/mL
- RAL start with a suppressed VL (SVL group): Started RAL from a bVL$\leq$200 copies/mL

All analyses were conducted separately in these two groups, as clinical experience and the previous literature (Table 4.4) indicated that it was likely that the reason for starting RAL and consequent virological outcomes would differ in these two groups. Baseline VL was approximated using any VL measurement taken before baseline, with no time restrictions. However, this was evaluated in a sensitivity analysis. Individuals with a VL recorded as below the detection limit of a VL higher than 200 (eg, 499 for 500) were classed as starting RAL with a raised VL, although different cut-offs were also evaluated in sensitivity analyses.

Figure 4.4. Selection of participants in EuroSIDA



The virological outcome I chose to study was VF, and I describe both time to and risk factors for VF. VF was defined differently according to VL at the time of RAL start, and was defined using both VL values and considering the discontinuation of drugs as indicative of VF.

- RAL start with a raised VL (RVL group): Confirmed VL>200 after at least 6 months of receiving RAL ("failure to supress")
- RAL start with a suppressed VL (SVL group): Confirmed VL>200 ("viral rebound")
- For both groups: Discontinuation of RAL or a drug used with RAL with the main reason for stopping given by the treating physician as VF

If an individual met more than one VF definition, the earliest date for VF was used. For the purposes of sample selection (Section 4.3.2 below) VF was considered to be on-going until RAL was stopped or VL declined to below 200 copies/ml, whichever occurred first. I chose to use a 200 copies/ml cut-off for the main analyses as EuroSIDA collects data from some countries that are relatively resource poor, and may still be using genotyping technology with this higher cut-off point, particularly at early follow-up times included in this analysis. The date of failure was put to the first of the two consecutive VL measures.

4.3.2. Resistance data and sample selection

I calculated a baseline GSS for the drugs received at the time of staring RAL using the ANRS (2015) interpretation rules. As the inclusion criteria guaranteed that individuals did not have any prior INSTI exposure, the GSS was calculated only for the rest of the regimen, defined as all drugs excluding RAL (564). Existing data on integrase resistance is limited in the EuroSIDA database, and I therefore selected a number of stored plasma samples to be retrospectively genotyped at the EuroSIDA central laboratory in Badalona, Spain. The samples were selected according to the following rules:

1. The date of the sample occurred between the first date of RAL failure and the last date of RAL failure

   or

2. The date of the sample occurred up to 3 months before the first date of RAL failure given that VL>50 copies/ml

   or

3. The date of the sample occurred up to 3 months after the last date of RAL failure given that VL>50 copies/ml

When more than one sample could be identified for a patient, I chose to include the earliest possible sample given that the viral load at the time of the sample was >50 copies per mL. Fifty copies/ml was considered the lowest VL it was considered feasible to genotype from (565–567). The principles were laid out in collaboration with virological experts and approved by the EuroSIDA steering committee.

4.3.2.1. Sequencing

Identified samples were sent to Badalona from the EuroSIDA plasma repository at the University of Copenhagen by the EuroSIDA coordinating centre staff in June 2015. They were sequenced using the Illumina Miseq platform, which is an NGS technology. The principles of NGS are described in more detail in Chapter 1. The reads generated by sequencing were aligned to the HIV-1HXB2 clonal sequence (GenBank ID: K03455|HIVHXB2CG). The Illumina platform uses paired-end sequencing, which involves both forward and reverse reads starting from the 3' and 5' end of the RNA strands respectively. To ensure that mutants were well balanced, the ratio of the forward and reverse reads should be close to 1. The minimum number of reads ("coverage") required to call a base using Illumina was estimated at 500 (R.Paredes, personal communication). Median (IQR) ratios of forward/reverse reads and total coverage is reported as an indication of the quality of the genotyping.

The main benefit of using an NGS platform is the ability to study minority variants, commonly defined as viral strains present at frequencies that are not detectable by Sanger sequencing. For the purposes of this analysis, this was defined as a frequency between 1-25% (568). 1% is chosen as the lower cut-off to remove variants detected a very low level and likely to be false positive detections.

4.3.3. Statistical methods

Patients' characteristics at baseline were compared between the raised viral load (RVL) and suppressed viral load (SVL) groups using the basic statistical inference techniques outlined in Chapter 2. The proportion of individuals receiving RAL was described per year and geographical region, using all individuals in EuroSIDA on ART with at least one RNA measurement in a given year as the denominator. The cumulative risk of experiencing VF to RAL was described using Kaplan Meier plots, and risk factors for failing RAL identified using Cox Proportional Hazards models.

In accordance with prior research (549), two different analyses were conducted, an intention to treat (ITT) analysis and one per protocol (PP) analysis. In the ITT analysis, individuals were censored at their last available clinic visit date and any drug switches were ignored. In the PP

analysis, individuals were censored at their last available clinic visit date or when RAL was stopped, whichever came first. These analyses answer different questions, both of which were considered to be of clinical importance. The ITT analysis presents estimates of the risk of VF after an individual has been prescribed RAL, whereas the PP analysis estimates the risk of VF while an individual is receiving RAL. For both the ITT and PP analysis, the last visit date was defined as the maximum visit date, date of RNA or CD4 measurement or ART start/stop date. Kaplan Meier plots were only drawn until less than 30 individuals remained in the risk set, which occurred after year 7. No events occurred in this period for either analysis group. Factors associated with successful genotyping were identified using the chi-squared test/kruskal wallis and logistic regression as appropriate. Only a descriptive analysis of the results from the genotyping is presented due to low sample size of patients with resistance tests.

4.3.3.1.Variable categorisation and missing data

The risk factors for VF evaluated in this analysis were identified based on the results from the literature review and the opinions of clinical experts. The selected variables, together with their final categorisations, can be seen in Table 4.5.

Table 4.5 Categorisation of potential risk factors for VF		
Variable	**Categories**	**Time-updated**
Gender	Female; Male	No
Ethnicity	White, Non-white	No
Age	Continuous (per 10 years)	No
Risk Group	MSM; PWID; Heterosexual; Other	No
Geographical Region[1]	Southern, Central West, Northern, Central East, Eastern	No
Co-infection with Hepatitis B (Ever positive for surface antigen (HBVGS))	Yes, No, Missing	No
Co-infection with Hepatitis C (Ever antibody positive)	Yes, No, Missing	No
Baseline VL[2,3]	Continuous, $\log_{10}$ scale higher	No
Baseline CD4[3]	Continuous, per 100 cells/mm^3	No
Baseline triple class resistance (resistance to NRTI, NNRTI and PI drugclasses)	Yes, No	No
HIV Subtype	B, Non-B, Unknown	No
Mono or dual therapy	Yes, No	No
Calendar Year of RAL start	Continuous, per more recent year	No

1. For the analysis of risk factors among individuals starting RAL with a suppressed VL, limited numbers meant that East and Central East had to be considered together. Argentina was considered together with Southern Europe as per EuroSIDA standard practice, as there were not enough individuals in each group to fit the model with separate categories.

2. Not considered for the analysis of risk factors among individuals starting RAL with a suppressed VL.

3. Any measurement prior to baseline was used to estimate these values. In sensitivity analyses, a shorter time-window (12 months) for defining baseline VL was used.

Missing data for the main outcome measurement, VL, was handled using a last observation carried forward (LOCF) approach, although this was evaluated in sensitivity analyses.

4.3.4. Sensitivity analyses

I conducted the following sensitivity analysis to assess the impact of some of the assumptions:

1) Censoring individuals FU-time 6 months after a VL measurement until a new VL measurement was taken. Individuals re-entered the analysis when a new VL measurement was taken

2) Defining VF as a confirmed VL>50 (for the SVL group), a confirmed VL>50 after at least 6 months of RAL (for the RVL group) or a physician indicated stop of RAL due to VF (regardless of group)

3) Same as (2) but using the threshold of 500 copies/mL for VF

4) Individuals with a baseline VL taken more than 12 months before baseline were excluded from the analysis

5) Individuals with less than 2 VL measurements available after their baseline VL measurement were excluded from the analysis

When the definitions of outcomes 2) and 3) were adopted, the cut off for the baseline value of VL used to define the SVL and RVL groups was changed accordingly. I was not able to use the limit of detection to do a sensitivity analysis as in Chapter 3, as this data was unfortunately not available in later versions of the EuroSIDA database due to changes to the data collection forms. For simplicity, only the results from the ITT analysis are shown for the sensitivity analyses.

4.4. Results

4.4.1. Use of raltegravir and baseline characteristics

In total, 2,447 individuals were included in the analyses. Those who started RAL but who were nonetheless excluded due to missing baseline VL or FU data were more likely to have Hepatitis C (p<0.001), have unknown Hepatitis B status (p<0.001), be injecting drug users (p<0.001) and they were also slightly more likely to originate from Southern, Central Eastern or Eastern Europe (p=0.006). They were also likely to be younger, have lower baseline CD4 counts and higher baseline VL values compared to those who were included (all p<0.0001).

Of those 2,447 included, the majority (1,642, 67%) started RAL with a suppressed VL (SVL group). Although EuroSIDA does not collect reasons for starting drugs, overall, 1584 (64.7%) of individuals had a reason for stopping a previous drug given in the month before starting RAL. A breakdown of these reasons is displayed according to the stratification groups in Table 4.6.

Table 4.6. Reasons for discontinuation a drug before starting RAL

Reason	Suppressed bVL (SVL)	Raised bVL (RVL)
	N (%)	N (%)
Treatment Failure	80 (7.1)	264 (57.5)
Abnormal Fat Redistribution	63 (5.6)	6 (1.3)
Concern of cardiovascular disease	28 (2.5)	4 (0.9)
Dyslipidaemia	74 (6.6)	4 (0.9)
Cardiovascular disease	22 (2.0)	1 (0.2)
Hypersensitivity Reaction	3 (0.3)	6 (1.3)
Toxicity, predominantly abdomen/GI tract	35 (3.1)	6 (1.3)
Toxicity - GI tract	46 (4.1)	9 (2.0)
Toxicity – Liver	31 (2.8)	9 (2.0)
Toxicity – Pancreas	3 (0.3)	2 (0.4)
Toxicity, predominantly CNS	28 (3.4)	5 (1.1)
Toxicity, predominantly kidneys	85 (7.6)	2 (0.4)
Toxicity, predominantly endocrine	4 (0.4)	
Diabetes	4 (0.4)	1 (0.2)
Haematological toxicity	5 (0.4)	2 (0.4)
Hyperlactataemia/lactic acidosis	2 (0.2)	
Toxicity, any other	69 (6.1)	16 (3.5)
Patient's choice	59 (5.2)	8 (1.7)
Physician's choice	302 (26.8)	70 (15.3)
Structured Treatment interruption	4 (0.4)	1 (0.2)
Other, not specified	149 (13.2)	42 (9.2)

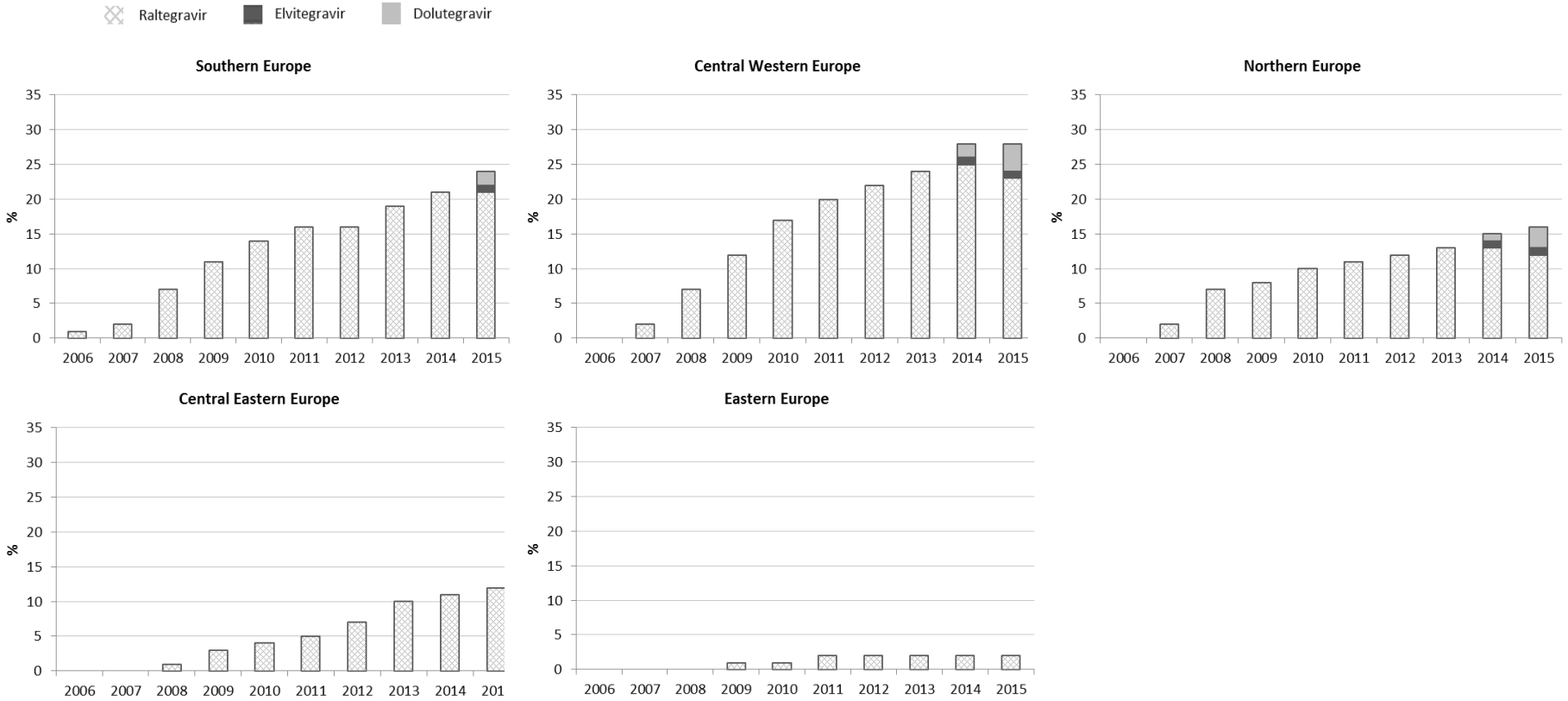
Among those in the SVL group, the most common reason for stopping a drug close to the date of RAL start was 'physician's choice' (26.8%), although when considering all toxicities together, 48% were indicated to have stopped because of toxicity. It is conceivable that most of those

reporting ‘physicians’ choice’ were switches for simplification in people with suppressed VL. As expected, treatment failure was the most common reason for stopping a drug among those starting RAL in the RVL group (57.5%).

The use of RAL increased significantly over time in all geographical regions (chi-squared test for trend p<0.0001, all regions), although the increase was more marked in Southern, Central Western and Northern Europe. RAL use showed an increase over time in Central Eastern and Eastern Europe, but the absolute number of individuals on cART receiving RAL in these regions was low, particularly in Eastern Europe (Figure 4.5). For completeness, the frequency of use of other drugs in the INSTI class is also shown in Figure 4.3. The use of ELV and DTG remained low across the study period, although DTG use increased significantly between 2014 and 2015 in Southern, Central Western and Northern Europe.

Baseline characteristics of the included participants according to bVL group can be seen in Table 4.7. Individuals in the RVL group were more likely to be female (p=0.003), originate from Central East or Eastern Europe (p<0.001) and to have acquired HIV through injecting drug use or heterosexual sex (p=0.003). They were also likely to be younger (p<0.001) and have started RAL in an earlier calendar year (p<0.001) than those in the SVL group. By definition, individuals in the RVL group higher bVL values (p<0.001), lower baseline CD4 counts (p<0.001) and lower CD4 count nadir (p=0.003) compared to those in the SVL group.

Figure 4.5 Use of integrase inhibitors over time in EuroSIDA, by geographical region[1]



1. *The proportion of individuals on each INSTI shown as a proportion of individuals under FU on ART in a given year, defined as having an RNA measurement taken in that particular year and being on at least 1 drug*

Table 4.7. Baseline characteristics of included patients

		All	RVL	SVL	P
		N (%)	N (%)	N (%)	
Total		2,377 (100)	805 (32.9)	1642 (67.1)	
Gender	Male	1,809 (76.1)	571 (72.4)	1238 (78.0)	0.003
	Female	568 (23.9)	218 (27.6)	350 (22.0)	
Ethnicity	White	2,017 (85.6)	656 (83.7)	1361 (86.5)	0.064
	Not White	340 (14.4)	128 (16.3)	212 (13.5)	
Region	South	794 (32.4)	273 (33.9)	520 (31.7)	<.001
	Central West	944 (38.6)	260 (32.3)	684 (41.7)	
	Northern	427 (17.5)	144 (17.9)	283 (17.2)	
	Central East	240 (9.8)	105 (13.0)	135 (8.2)	
	East	43 (1.7)	23 (2.9)	20 (1.2)	
Risk Group	MSM	983 (41.7)	289 (36.8)	694 (44.1)	0.003
	PWID	554 (23.5)	204 (26.0)	350 (22.2)	
	Heterosexual	645 (27.3)	237 (30.2)	408 (25.9)	
	Other	178 (7.5)	55 (7.0)	123 (7.8)	
Baseline GSS	>3	968 (39.6)	362 (45.0)	592 (36.1)	<.001
	<3	112 (4.6)	61 (7.6)	65 (4.0)	
	Unknown	1,367 (55.9)	382 (47.5)	985 (60.0)	
Subtype	B	831 (34.0)	303 (37.6)	528 (32.2)	<.001
	Not B	154 (6.3)	66 (8.2)	88 (5.4)	
	Unknown	1,462 (59.8)	436 (54.2)	1026 (62.5)	
Hepatitis C	No	1,254 (51.3)	401 (49.8)	853 (51.9)	<.001
	Yes	1,072 (43.8)	344 (42.7)	728 (44.3)	

	Unknown	121 (4.9)	60 (7.5)	61 (3.7)	
Hepatitis B	No	1,966 (80.3)	616 (76.5)	1350 (82.2)	0.003
	Yes	122 (5.0)	44 (5.5)	78 (4.8)	
	Unknown	359 (14.7)	145 (18.0)	214 (13.0)	
History of mono/dual therapy	No	1,560 (63.8)	530 (65.8)	1034 (63.0)	0.165
	Yes	887 (36.3)	275 (34.2)	608 (37.0)	
		Median (IQR)	**Median (IQR)**	**Median (IQR)**	
Age	Years	49 (44-55)	47 (41 - 53)	50 (45 - 56)	<.001
Baseline VL	log_{10} cp/ml	49 (36-1,400)	10,528 (1,500 – 75,000)	39 (19 - 49)	<.001
Baseline CD4	cells/mm^3	441 (260-659)	277 (159 - 449)	525 (352 - 735)	<.001
Nadir CD4	cells/mm^3	113 (38-205)	100 (31 - 193)	120 (42 - 210)	0.003
Month/Year of RAL start	Year	10/10 (01/09-11/12)	11/09 (06/08-(11/11)	04/11 (06/09-03/13)	<.001

4.4.2. Baseline treatment and resistance patterns.

A large number of different treatment combinations were used: 778 different combinations in total. The most common treatment regimen was RAL + Tenofovir (TDF) and Emtricitabine (FTC) among both patient groups (6.4 and 8.6% in the RVL and SVL groups respectively (Table 4.8).

Table 4.8. Most common treatment combinations used with RAL

Regimen	N	%
Among patients with a raised baseline VL		
Tenofovir/Emtricitabine	105	6.39
Tenofovir/Emtricitabine/Darunavir(r)	72	4.38
Lamivudine/Abacavir	68	4.14
Among individuals with a supressed baseline VL		
Tenofovir/Emtricitabine	69	8.57
Darunavir (r)/ Etravirine	27	3.35
Darunavir (r)	26	3.23

Drugs used before and after RAL can be seen in Table 4.9 below.

Table 4.9. Drugs given before and with RAL at baseline

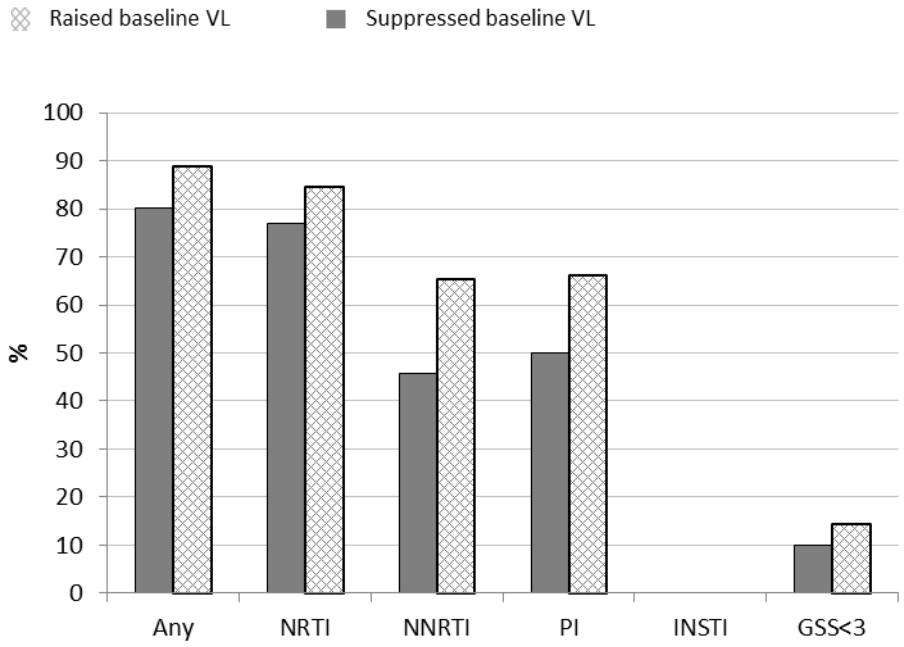
	RVL	SVL
Before baseline	N (%)	N (%)
NRTI	1374 (83.7)	590 (73.3)
NNRTI	480 (29.2)	143 (17.8)
PI (any)	1146 (69.8)	472 (58.6)
LPV/r	355 (21.6)	148 (18.4)
DRV/r	282 (17.2)	90 (11.2)
T20	91 (5.5)	34 (4.2)
Number of drugs (Median, IQR)	3 (3-3)	3 (3-3)
At baseline	N (%)	N (%)
NRTI	1429 (87.0)	680 (84.5)
NNRTI	536 (32.6)	283 (35.2)
PI (any)	1105 (67.3)	595 (73.9)
Lopinavir (r)	313 (19.1)	142 (17.6)
Darunavir (r)	445 (27.1)	366 (45.5)
T20	75 (5.6)	67 (8.3)
Number of drugs (Median, IQR)	4 (3-6)	4 (3-5)

1. *Before baseline describes drugs given the day before RAL was started*

The majority of individuals in both patient groups were on PI-containing cART both before and after initiation of RAL. The median number of drugs used increased from 3 (3-3) in both groups to 4 (3-5) and 4 (3-6) in the RVL and SVL groups respectively at baseline. RAL was commonly paired with Darunavir (DRV), particularly among individuals in the SVL group (45.5%).

1080 (44%) of individuals had a resistance test result at baseline; 423 (53%) in the RVL and 530 (32%) in the SVL group. The resistance test was done a median of 5.3 years (IQR=2.1-8.9) before the start of RAL (7.1 (4.2-10.2) and 2.3 (0.3-5.9) among the SVL and RVL groups respectively). Out of those tested for resistance, the vast majority had any detected resistance in both groups (Figure 4.6). However, most were also treated with regimens that had at least two active drugs (90.1 and 85.6 in the SVL and RVL groups respectively).

Figure 4.6. Resistance patterns at baseline according to RVL/SVL RAL use



A total of 401 (16.4%) of patients had resistance to all 3 major drug classes, 203 (25.2%) of RVL patients and 198 (12.1%) of SVL patients.

4.4.3. Time to and risk factors for virological failure

In total, 262 individuals experienced VF in the ITT analyses and 192 individuals in PP analyses. The majority of VF's occurred in the RVL group (58% in ITT and 61% in PP analyses). People in the RVL group were also followed-up for longer: a median of 3.9 (1.7-5.8, ITT) and 2.8 (0.8-5.2, PP) years and those in the SVL group for 3.1 (1.5-5.0, ITT) and 2.4 (1.0-4.5, PP) years respectively. KM estimates with 95% CI of time to VF according to bVL group can be seen in Figure 4.7 below. The cumulative probability of experiencing VF by 7 years was estimated at 12.0% (95%CI=8.4-16.8) (ITT) and 8.7% (95%CI=5.6-12.6) (PP) for individuals in the SVL group. These estimates were two-fold higher for those in the RVL group: 24.8% (19-32) (ITT) and 22.2% (16.3-29.9) (PP) respectively. As expected, estimates from the ITT analyses were higher than those form the PP analyses. Of those who did fail, the median VL at failure was relatively low: 2,119 (406-24,5999 copies/ml in the RVL group and 361 (39-8,260) copies/ml in the SVL group.

Risk factors associated with failing RAL can be seen in Table 4.10 (SVL group) and Table 4.11 (RVL group). Among those in the SVL group, a low number of events made it challenging to identify any risk factors for VF. However, there was some evidence that individuals with higher baseline CD4 counts were less likely to experience VF (aHR=0.92, 95%CI=0.85-0.99), although the evidence supporting this finding was weak (p=0.04). There was also an indication that the probability of RAL failure decreased with calendar time of starting RAL (aHR=0.89, 95%CI=0.79-1.01, p=0.077). Although the HR's were similar in both ITT and PP analyses, the weak evidence associated with both of these findings was even more attenuated in the PP analyses (p=0.17 and p=0.2 respectively), possibly as a result of the lower number of events included in the PP analysis.

Figure 4.7. Time to VF following RAL start in ITT (a) and PP (b) analyses

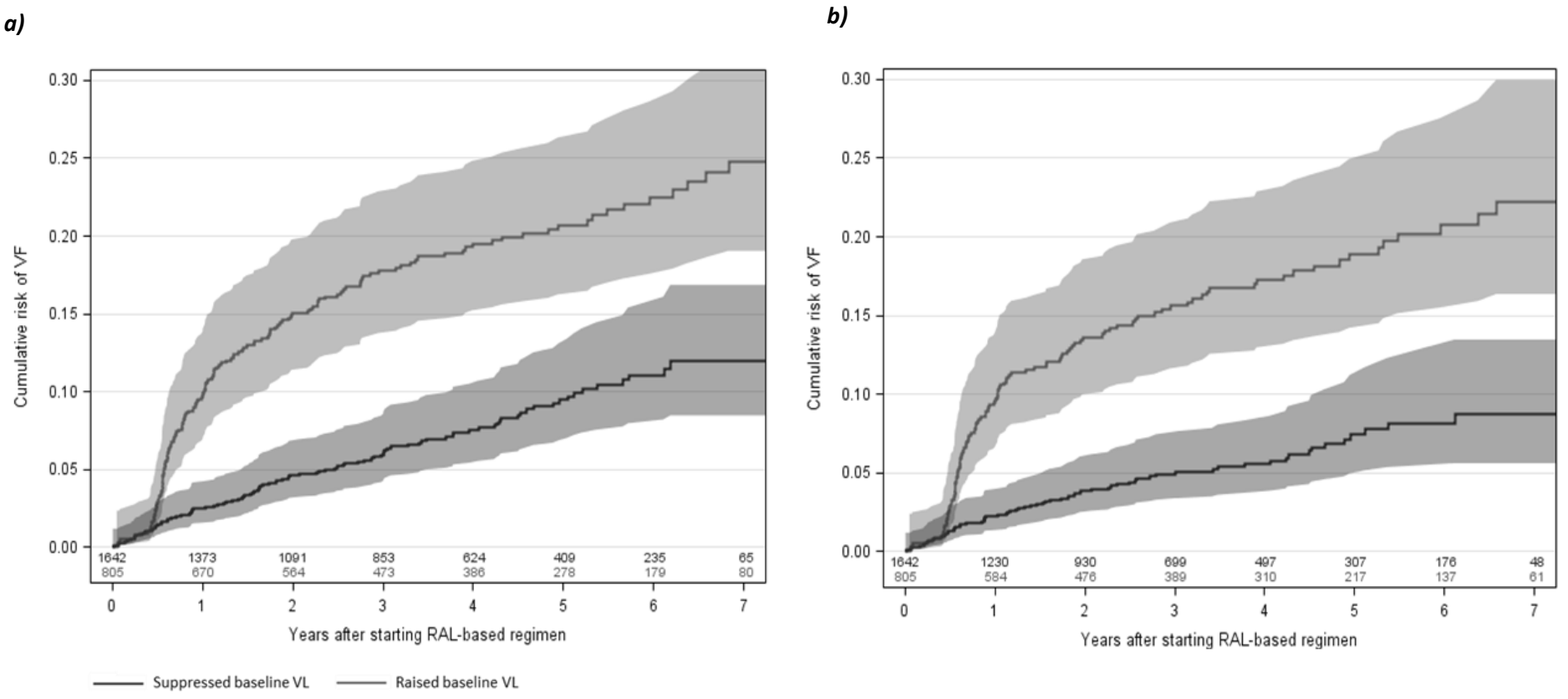


Table 4.10. Factors associated with failing RAL among patients in the SVL group

		ITT		PP	
		aHR (95% CI)	**P**	**aHR (95% CI)**	**P**
Gender	Male				
	Female	1.06 (0.61 - 1.85)	0.835	0.83 (0.42 - 1.65)	0.600
Age	Per 10 years older	0.85 (0.69 - 1.05)	0.139	0.86 (0.67 - 1.12)	0.265
Ethnicity	White				
	Not White	0.66 (0.34 - 1.28)	0.217	0.57 (0.25 - 1.28)	0.173
Region	South		0.895		0.719
	Central West	1.11 (0.67 - 1.84)		1.29 (0.70 - 2.38)	
	Northern	0.94 (0.51 - 1.74)		1.02 (0.47 - 2.19)	
	Central East and East	0.86 (0.35 - 2.10)		0.78 (0.26 - 2.33)	
Risk Group	MSM		0.215		0.335
	PWID	1.21 (0.65 - 2.26)		1.49 (0.69 - 3.22)	
	Heterosexual	0.87 (0.47 - 1.60)		1.27 (0.63 - 2.57)	
	Other	1.78 (0.93 - 3.41)		2.03 (0.93 - 4.44)	
Baseline CD4	Per 100 cells/mm^3 higher	0.92 (0.85 - 0.99)	0.037	0.94 (0.85 - 1.03)	0.171
CD4 nadir	Per 100 cells/mm^3 higher	1.15 (0.96 - 1.38)	0.122	1.10 (0.88 - 1.38)	0.414
Subtype	B		0.278		0.532
	Not B	1.07 (0.47 - 2.47)		1.22 (0.45 - 3.32)	
	Unknown	0.71 (0.45 - 1.12)		0.78 (0.44 - 1.36)	
Triple Class Resistance	No		0.432		0.229
	Yes	1.23 (0.73 - 2.06)		1.46 (0.79 - 2.69)	
History of mono or dual therapy	No		0.980		0.215
	Yes	1.00 (0.67 - 1.47)		0.73 (0.45 - 1.20)	

Hepatitis B	No		0.502		0.919
	Yes	0.74 (0.34 - 1.62)		1.15 (0.35 - 3.71)	
	Unknown	0.53 (0.18 - 1.54)		0.96 (0.23 - 4.09)	
Hepatitis C	No		0.893		0.885
	Yes	0.93 (0.59 - 1.47)		1.04 (0.59 - 1.84)	
	Unknown	0.77 (0.22 - 2.69)		0.71 (0.15 - 3.31)	
Calendar Year of RAL start	Per more recent year	0.89 (0.79 - 1.01)	0.071	0.91 (0.79 - 1.05)	0.200

Risk factors for VF were different among those in the RVL group. Among this group, individuals were more likely to experience VF if they were of a non-white ethnicity (aHR=1.92 comparing non-white vs. white, 95%CI=1.18 - 3.10, p=0.008), receiving care in central eastern or eastern Europe (aHR=2.29 compared to Southern Europe, 95%CI=1.34-3.90 and aHR=3.22, 95%CI=1.39-7.46 respectively, p<0.001). There were also differences according to baseline VL and CD4 levels: individuals with higher baseline VL levels were more likely to experience VF (aHR=1.22 per $\log_{10}$ higher, 95%CI=1.01-1.46, p=0.04), whereas individuals with higher nadir CD4 counts were less likely to experience VF (aHR=0.82 per 100 cells higher, 95%CI=0.66-1.01), although the evidence supporting the latter was weak (p=0.07). The results also suggested that individuals with diagnosed Hepatitis C were less likely to experience VF on RAL compared to those with no Hepatitis C (aHR=0.57, 95%CI=0.36-0.90, p=0.03). The adjusted hazard ratio for calendar year indicated that the risk of VF had decreased with later years of RAL start in a similar manner to that seen among individuals who started RAL with a suppressed bVL (aHR=0.90, 95%CI=0.80-1.01), although this difference could be due to chance (p=0.07).

Some of the results of the PP analysis were different from those found in the ITT analysis. Geographical region, CD4 nadir and hepatitis C remained associated with VF, and the association between the risk of VF and calendar time grew slightly stronger (aHR=0.88, 0.78-1.00, p=0.04). However, there was no evidence to support an association with ethnicity and baseline VL.

Table 4.11. Factors associated with failing RAL among patients in the RVL group

Multivariable		ITT		PP	
		aHR (95% CI)	**P**	**aHR (95% CI)**	**P**
Gender	Male		0.383		0.295
	Female	0.82 (0.53 - 1.28)		0.76 (0.46 - 1.27)	
Age	Per 10 years older	0.83 (0.67 - 1.02)	0.076	0.82 (0.65 - 1.03)	0.093
Ethnicity	White		0.008		0.336
	Not White	1.92 (1.18 - 3.10)		1.33 (0.74 - 2.38)	
Region	South		<.001		0.002
	Central West	0.81 (0.50 - 1.31)		0.81 (0.47 - 1.40)	
	Northern	0.72 (0.42 - 1.24)		0.84 (0.46 - 1.54)	
	Central East	2.29 (1.34 - 3.90)		2.53 (1.39 - 4.60)	
	East	3.22 (1.39 - 7.46)		3.05 (1.22 - 7.63)	
Risk Group	MSM		0.048		0.047
	PWID	2.11 (1.22 - 3.64)		2.28 (1.22 - 4.23)	
	Heterosexual	1.39 (0.85 - 2.27)		1.55 (0.88 - 2.73)	
	Other	1.85 (0.94 - 3.64)		2.13 (1.03 - 4.43)	
Baseline VL	Per log_{10} cp/ml higher	1.22 (1.01 - 1.46)	0.035	1.10 (0.89 - 1.36)	0.381
Baseline CD4	Per 100 cells/mm^3 higher	0.92 (0.84 - 1.02)	0.125	0.94 (0.84 - 1.05)	0.243
CD4 nadir	Per 100 cells/mm^3 higher	0.82 (0.66 - 1.01)	0.065	0.79 (0.62 - 1.01)	0.059
Subtype	B		0.124		0.626
	Not B	0.76 (0.41 - 1.41)		0.70 (0.33 - 1.48)	
	Unknown	0.65 (0.42 - 0.99)	0.689	0.89 (0.55 - 1.43)	0.796
Triple Class Resistance	No TCR				
	TCR	0.92 (0.59 - 1.41)	0.532	0.94 (0.57 - 1.53)	0.198

History of mono or dual therapy	No				
	Yes	1.12 (0.78 - 1.61)		1.31 (0.87 - 1.97)	
Hepatitis B	No		0.007		0.026
	Yes	2.35 (1.28 - 4.32)		1.90 (0.92 - 3.91)	
	Unknown	0.61 (0.30 - 1.23)		0.44 (0.19 - 1.01)	
Hepatitis C	No		0.035		0.030
	Yes	0.57 (0.36 - 0.90)		0.55 (0.33 - 0.93)	
	Unknown	1.09 (0.46 - 2.60)		1.42 (0.54 - 3.76)	
Calendar Year of RAL start	Per more recent year	0.90 (0.81 - 1.01)	0.064	0.88 (0.77 - 1.00)	0.043

4.4.4.Sensitivity analyses

A number of sensitivity analyses were performed by varying the inclusion criteria and the definition of VF as described in the methods. The number of failure events and FU time included as a result of changing these definitions in the various can be seen in Table 4.12Table 4.16. As expected, more failure events were detected when using the lower VL cut-off of 50 copies/mL compared to the 200 copies/mL threshold used in the main analysis. The results of the sensitivity analyses were very similar to those found in the main analysis. For those with a suppressed bVL (Table 4.13), the findings indicated borderline associations with baseline CD4 and calendar year in all these alternative analyses. For individuals starting RAL with a raised bVL (Table 4.15), there were some differences in terms of statistical significance across sensitivity analyses for ethnicity, baseline VL, CD4 nadir and calendar year, although the aHR were all similar. The most consistent finding across all analyses was a higher risk of VF in central eastern and eastern Europe as compared to southern Europe.

Table 4.12. Number of patients, FU time and VFs with a raised and supressed viral load under different assumptions

		Main analysis	Sensitivity 1	Sensitivity 2	Sensitivity 3	Sensitivity 4	Sensitivity 5
		Cut-off: 200	Cut-off: 50	Cut-off: 500	Censor: 6m	Baseline VL taken <12 m of baseline	At least 2 VL measures after baseline
			N (%)	N (%)	N (%)	N (%)	N (%)
Total		2,447	2,447	2,447	2,447	2,213	2,214
N (sup.)	*N (%)*[1]	1,642 (67.1)	1,453 (59.4)	1,741 (71.2)	1,642 (67.1)	1,557 (70.4)	1,495 (67.5)
N (not sup.)	*N (%)*[1]	805 (32.9)	994 (40.6)	706 (28.9)	805 (32.9)	656 (29.6)	719 (32.5)
ITT							
FU (sup.)	*Median (IQR)*	3.1 (1.5-5.0)	2.9 (1.3-4.8)	3.2 (1.5-5.1)	2.8 (1.4-4.5)	3.1 (1.5-5.1)	3.4 (1.9-5.2)
FU (not sup.)	*Median (IQR)*	3.9 (1.7-5.8)	2.8 (1.0-5.2)	4.0 (1.7-5.9)	3.0 (1.3-5.0)	3.8 (1.5-5.8)	4.1 (1.9-6.0)
Failures (sup.)	*N (%)*[2]	110 (12.0)	173 (28.3)	100 (10.6)	110 (12.4)	106 (12.0)	110 (11.9)
Failures (not sup.)	*N (%)*[2]	152 (24.8)	307 (41.2)	118 (22.4)	152 (26.4)	139 (27.1)	150 (27.1)
PP							
FU (sup.)	*Median (IQR)*	2.4 (1.0-4.5)	2.3 (0.9-4.3)	2.5 (1.0-4.5)	2.2 (1.0-3.9)	2.4 (1.0-4.5)	2.7 (1.3-4.7)
FU (not sup.)	*Median (IQR)*	2.8 (0.8-5.2)	2.2 (0.6-4.6)	3.1 (0.9-5.4)	2.2 (0.8-4.3)	2.7 (0.8-5.3)	3.2 (1.0-5.4)
Failures (sup.)	*N (%)*[2]	74 (8.7)	142 (30.1)	64 (7.4)	74 (8.8)	72 (8.9)	74 (8.8)
Failures (not sup.)	*N (%)*[2]	118 (22.3)	251 (38.6)	87 (19.0)	118 (24.3)	110 (25.1)	118 (23.3)

1. *As a proportion of the total*
2. *KM proportion at end of FU*

Table 4.13. Factors associated with failing RAL among patients with a suppressed baseline VL, sensitivity analyses (ITT)

		Sensitivity 1 Cut-off: 50		Sensitivity 2 Cut-off: 500		Sensitivity 3 Censor: 6m	
		aHR (95% CI)	**P**	**aHR (95% CI)**	**P**	**aHR (95% CI)**	**P**
Gender	Male						
	Female	0.70 (0.44 - 1.12)	0.139	0.92 (0.51 - 1.64)	0.769	1.07 (0.61 - 1.87)	0.822
Age	Per 10 years	0.94 (0.79 - 1.13)	0.514	0.92 (0.74 - 1.16)	0.485	0.85 (0.69 - 1.05)	0.142
Ethnicity	White						
	Not White	0.88 (0.53 - 1.48)	0.637	1.03 (0.57 - 1.87)	0.926	0.66 (0.34 - 1.28)	0.221
Region	South		0.244		0.660		0.903
	Central West	0.73 (0.49 - 1.07)		1.26 (0.75 - 2.14)		1.10 (0.67 - 1.82)	
	Northern	0.64 (0.39 - 1.05)		1.03 (0.55 - 1.95)		0.92 (0.50 - 1.70)	
	Central East and East	0.70 (0.33 - 1.48)		0.73 (0.25 - 2.15)		0.88 (0.36 - 2.15)	
Risk Group	MSM		0.343		0.142		0.241
	PWID	1.41 (0.87 - 2.28)		1.31 (0.67 - 2.53)		1.22 (0.65 - 2.27)	
	Heterosexual	1.05 (0.65 - 1.70)		0.89 (0.47 - 1.69)		0.87 (0.47 - 1.60)	
	Other	1.53 (0.85 - 2.73)		1.95 (1.00 - 3.77)		1.74 (0.90 - 3.33)	
Baseline CD4	Per 100 cells/mm^3 higher	0.95 (0.89 - 1.01)	0.085	0.91 (0.83 - 0.99)	0.025	0.92 (0.84 - 1.00)	0.038
CD4 nadir	Per 100 cells/mm^3 higher	1.04 (0.90 - 1.20)	0.574	1.19 (0.98 - 1.44)	0.073	1.15 (0.96 - 1.38)	0.124
Subtype	B		0.357		0.213		0.297
	Not B	0.82 (0.40 - 1.71)		1.13 (0.51 - 2.52)		1.07 (0.46 - 2.46)	

	Unknown	0.76 (0.53 - 1.11)		0.68 (0.42 - 1.10)		0.72 (0.46 - 1.13)	
Triple Class Resistance	No		0.677		0.541		0.411
	Yes	1.10 (0.71 - 1.70)		0.84 (0.48 - 1.48)		1.24 (0.74 - 2.08)	
History of mono or dual therapy	No		0.891		0.727		0.963
	Yes	1.02 (0.74 - 1.41)		0.93 (0.61 - 1.40)		0.99 (0.67 - 1.47)	
Hepatitis B	No		0.480		0.443		0.512
	Yes	0.68 (0.36 - 1.28)		0.62 (0.29 - 1.30)		0.73 (0.33 - 1.59)	
	Unknown	0.75 (0.34 - 1.64)		0.62 (0.22 - 1.72)		0.53 (0.18 - 1.55)	
Hepatitis C	No		0.362		0.862		0.904
	Yes	0.87 (0.59 - 1.28)		1.03 (0.64 - 1.68)		0.92 (0.58 - 1.47)	
	Unknown	1.48 (0.68 - 3.20)		0.73 (0.21 - 2.58)		0.79 (0.23 - 2.78)	
Calendar Year of RAL	Per Year	0.86 (0.78 - 0.94)	0.002	0.83 (0.73 - 0.95)	0.008	0.89 (0.79 - 1.00)	0.057

Table 4.14. Factors associated with failing RAL among patients with a suppressed baseline VL, sensitivity analyses (ITT)

		Sensitivity 4 Baseline VL taken <12 m of baseline		Sensitivity 5 At least two VL after baseline	
		aHR (95% CI)	**P**	**aHR (95% CI)**	**P**
Gender	Male				
	Female	1.05 (0.60 - 1.86)	0.859	1.06 (0.61 - 1.86)	0.826
Age	Per 10 years	0.87 (0.70 - 1.08)	0.195	0.85 (0.69 - 1.06)	0.145
Ethnicity	White				
	Not White	0.68 (0.35 - 1.33)	0.259	0.66 (0.34 - 1.29)	0.223
Region	South		0.966		0.917
	Central West	1.06 (0.63 - 1.76)		1.10 (0.66 - 1.81)	
	Northern	0.97 (0.53 - 1.80)		0.94 (0.51 - 1.74)	
	Central East and East	0.85 (0.35 - 2.08)		0.86 (0.35 - 2.09)	
Risk Group	MSM		0.346		0.227
	PWID	1.20 (0.63 - 2.26)		1.22 (0.66 - 2.28)	
	Heterosexual	0.89 (0.48 - 1.64)		0.87 (0.47 - 1.61)	
	Other	1.67 (0.85 - 3.28)		1.76 (0.92 - 3.38)	
Baseline CD4	Per 100 cells/mm^3 higher	0.92 (0.85 - 1.00)	0.055	0.92 (0.85 - 0.99)	0.037
CD4 nadir	Per 100 cells/mm^3 higher	1.12 (0.94 - 1.35)	0.211	1.15 (0.96 - 1.38)	0.129
Subtype	B		0.226		0.279
	Not B	1.03 (0.45 - 2.39)		1.07 (0.46 - 2.46)	

	Unknown	0.68 (0.43 - 1.08)		0.71 (0.45 - 1.12)	
Triple Class Resistance	No		0.491		0.424
	Yes	1.20 (0.71 - 2.03)		1.23 (0.74 - 2.06)	
History of mono or dual therapy	No		0.815		0.969
	Yes	0.95 (0.64 - 1.43)		0.99 (0.67 - 1.47)	
Hepatitis B	No		0.724		0.513
	Yes	0.83 (0.36 - 1.92)		0.75 (0.34 - 1.63)	
	Unknown	0.64 (0.21 - 1.95)		0.53 (0.18 - 1.55)	
Hepatitis C	No		0.894		0.904
	Yes	0.92 (0.57 - 1.48)		0.93 (0.59 - 1.48)	
	Unknown	0.78 (0.22 - 2.74)		0.78 (0.22– 2.73)	
Calendar Year of RAL	Per Year	0.91 (0.80 - 1.03)	0.141	0.90 (0.80 - 1.02)	0.112

Table 4.15. Factors associated with failing RAL among patients with a raised baseline VL, sensitivity analyses (ITT)

		Sensitivity 1 Cut-off: 50		Sensitivity 2 Cut-off: 500		Sensitivity 3 Censor: 6m	
		aHR (95% CI)	**P**	**aHR (95% CI)**	**P**	**aHR (95% CI)**	**P**
Gender	Male		0.232		0.274		0.487
	Female	0.83 (0.60 - 1.13)		0.76 (0.46 - 1.25)		0.85 (0.55 - 1.33)	
Age	Per 10 years older	0.90 (0.78 - 1.03)	0.127	0.73 (0.58 - 0.94)	0.013	0.83 (0.67 - 1.02)	0.083
Ethnicity	White		0.088		0.349		0.013
	Not White	1.34 (0.96 - 1.88)		1.31 (0.75 - 2.29)		1.85 (1.14 - 2.99)	
Region	South		<.001		0.003		<.001
	Central West	0.87 (0.63 - 1.20)		0.82 (0.48 - 1.40)		0.85 (0.52 - 1.37)	
	Northern	0.78 (0.54 - 1.13)		0.60 (0.32 - 1.12)		0.74 (0.43 - 1.28)	
	Central East	1.32 (0.88 - 1.96)		1.87 (1.03 - 3.37)		2.38 (1.40 - 4.06)	
	East	2.86 (1.60 - 5.09)		3.46 (1.39 - 8.64)		3.13 (1.35 - 7.24)	
Risk Group	MSM		0.203		0.090		0.036
	PWID	1.43 (0.97 - 2.10)		2.10 (1.14 - 3.89)		2.16 (1.25 - 3.72)	
	Heterosexual	1.36 (0.97 - 1.90)		1.59 (0.92 - 2.73)		1.36 (0.83 - 2.23)	
	Other	1.31 (0.80 - 2.15)		1.18 (0.50 - 2.78)		1.92 (0.98 - 3.78)	
Baseline VL	log_{10} cp/ml higher	1.14 (1.02 - 1.28)	0.024	1.16 (0.93 - 1.45)	0.192	1.22 (1.02 - 1.46)	0.033
Baseline CD4	100 cells/mm^3 higher	0.99 (0.93 - 1.05)	0.778	0.93 (0.83 - 1.05)	0.234	0.92 (0.83 - 1.02)	0.108
CD4 nadir	100 cells/mm^3 higher	0.89 (0.78 - 1.02)	0.085	0.80 (0.63 - 1.02)	0.073	0.81 (0.65 - 1.00)	0.052
Subtype	B		0.030		0.264		0.196
	Not B	0.72 (0.46 - 1.15)		1.04 (0.53 - 2.05)		0.79 (0.43 - 1.46)	
	Unknown	0.68 (0.51 - 0.92)		0.69 (0.43 - 1.11)		0.68 (0.44 - 1.04)	

Triple Class Resistance	No TCR		0.505		0.569		0.663
	TCR	0.90 (0.67 - 1.22)		0.87 (0.53 - 1.41)		0.91 (0.59 - 1.40)	
History of mono or dual therapy	No		0.526		0.395		0.491
	Yes	1.08 (0.85 - 1.39)		1.19 (0.80 - 1.79)		1.13 (0.79 - 1.62)	
Hepatitis B	No		0.290		0.054		0.010
	Yes	1.37 (0.85 - 2.19)		2.00 (1.00 - 4.02)		2.33 (1.27 - 4.28)	
	Unknown	0.83 (0.53 - 1.30)		0.60 (0.27 - 1.30)		0.66 (0.32 - 1.36)	
Hepatitis C	No		0.144		0.107		0.044
	Yes	0.78 (0.57 - 1.06)		0.60 (0.36 - 1.01)		0.58 (0.37 - 0.91)	
	Unknown	1.21 (0.68 - 2.14)		1.18 (0.46 - 3.04)		1.09 (0.45 - 2.64)	
Calendar Year of RAL start	Per more recent year	0.93 (0.86 - 1.00)	0.064	0.87 (0.77 - 0.99)	0.038	0.90 (0.81 - 1.01)	0.070

Table 4.16. Factors associated with failing RAL among patients with a raised baseline VL, sensitivity analyses (ITT)

		Sensitivity 4 Baseline VL taken <12 m of baseline		Sensitivity 5 At least two VL after baseline	
		aHR (95% CI)	**P**	**aHR (95% CI)**	**P**
Gender	Male		0.861		0.509
	Female	0.96 (0.61 - 1.52)		0.86 (0.55 - 1.35)	
Age	Per 10 years older	0.79 (0.63 - 0.99)	0.039	0.81 (0.66 - 1.01)	0.058
Ethnicity	White		0.024		0.016
	Not White	1.80 (1.08 - 3.01)		1.82 (1.12 - 2.96)	
Region	South		<.001		<.001
	Central West	0.84 (0.51 - 1.40)		0.79 (0.49 - 1.29)	
	Northern	0.92 (0.53 - 1.63)		0.72 (0.42 - 1.25)	
	Central East	2.72 (1.55 - 4.76)		2.23 (1.30 - 3.82)	
	East	3.33 (1.42 - 7.81)		3.12 (1.35 - 7.24)	
Risk Group	MSM		0.020		0.067
	PWID	2.10 (1.20 - 3.70)		2.05 (1.19 - 3.56)	
	Heterosexual	1.08 (0.64 - 1.82)		1.32 (0.80 - 2.16)	
	Other	2.09 (1.05 - 4.15)		1.76 (0.89 - 3.47)	
Baseline VL	log_{10} cp/ml higher	1.26 (1.04 - 1.53)	0.018	1.21 (1.00 - 1.45)	0.045
Baseline CD4	100 cells/mm^3 higher	0.91 (0.82 - 1.01)	0.074	0.92 (0.83 - 1.02)	0.108
CD4 nadir	100 cells/mm^3 higher	0.79 (0.63 - 0.99)	0.044	0.82 (0.66 - 1.02)	0.079
Subtype	B		0.389		0.138
	Not B	0.85 (0.45 - 1.63)		0.77 (0.42 - 1.43)	
	Unknown	0.73 (0.47 - 1.14)		0.65 (0.42 - 1.00)	

Triple Class Resistance	No TCR		0.341		0.730
	TCR	0.81 (0.52 - 1.26)		0.93 (0.60 - 1.43)	
History of mono or dual therapy	No		0.391		0.477
	Yes	1.18 (0.81 - 1.72)		1.14 (0.79 - 1.64)	
Hepatitis B	No		0.021		0.007
	Yes	2.32 (1.25 - 4.28)		2.42 (1.31 - 4.46)	
	Unknown	0.82 (0.35 - 1.92)		0.62 (0.29 - 1.30)	
Hepatitis C	No		0.075		0.040
	Yes	0.60 (0.37 - 0.95)		0.58 (0.37 - 0.91)	
	Unknown	1.10 (0.41 - 2.94)		1.14 (0.46 - 2.80)	
Calendar Year of RAL start	Per more recent year	0.91 (0.81 - 1.02)	0.108	0.92 (0.82 - 1.03)	0.139

4.4.5.Resistance profiles at failure

No individual who experienced VF in this study had data on integrase resistance submitted to the EuroSIDA study. Thirty-three samples stored in the plasma repository were identified and sent for NGS genotyping. Individuals with stored plasma samples were more likely to originate from central eastern Europe ($p<0.0001$) and had lower baseline CD4 counts ($p<0.001$) compared to those experiencing failure without a stored sample. The characteristics of these samples can be seen in Table 4.17. Most samples selected were taken during RAL failure, although three samples occurred before the defined first date of failure and five samples after the first defined date of failure.

The overall genotyping success rate was low (11/33, 34%). Factors associated with successful genotyping can be seen in Table 4.18. The only factors associated with genotyping success was the VL at the time of sample (uOR=3.7, 95%CI=1.4-10.0 per $\log_{10}$ higher increase, p=0.009) and, marginally, VL at the time of VF (uOR=2.2, 95%CI=0.99-4.9 per $\log_{10}$ higher increase, p=0.05).

Table 4.17. Characteristics of samples and genotyping success

Patient	Sample Date	RNA	Sample taken before (B), After (A) or During (D) failure episode	Time between the date of first failure and sample date (months)	Time between RNA measurement date and sample date (days)	Success
1	03-Feb-09	4120	D	2.9	0	No
2	17-May-11	23000	D	0	0	Yes
3	30-May-11	351	D	0	0	No
4	06-Jan-11	287000	D	0	0	No
5	02-Dec-10	209000	B	-2.1	-23	Yes
6	04-Nov-13	382	D	0	0	No
7	12-Dec-11	262	D	14	-68	No
8	16-Jul-10	847	D	0	-27	No
9	27-Aug-13	397	D	31	0	No
10	03-Nov-09	21200	D	0	0	No
11	02-Jun-11	9360	D	5.5	0	Yes
12	16-Jun-10	1380	D	3.0	0	No
13	16-Jun-10	59100	D	0	0	Yes
14	09-Nov-10	690	D	3.2	0	No
15	10-Nov-09	29700	D	0	0	Yes
16	13-Jan-11	267	D	0	0	No
17	27-Aug-12	8260	D	2.8	-84	Yes
18	02-Dec-08	1790	D	1.4	0	No
19	02-Mar-11	73000	D	4.6	0	Yes
20	30-Jun-09	14774	D	3.0	-1	No
21	24-May-10	2517	D	0	0	Yes
22	20-May-11	270	A	1.7	-22	No
23	20-Dec-10	5400	A	4.5	0	No
24	22-Sep-10	17198	D	9.7	-35	Yes
26	31-May-12	498	D	2.8	-84	Yes
27	21-Apr-08	127	B	-1	-105	No
28	28-Feb-13	101	B	-2	0	No
29	29-Sep-09	552	A	1.9	-1	Yes
30	11-Feb-10	867	D	1.5	0	No
31	23-Jan-12	71	A	32.4	-7	No
32	21-Oct-08	284	A	2.1	-8	No
33	29-Feb-12	1680	D	0	0	No

Table 4.18. Factors associated with successful genotyping

		Total (N=32)	Successful (N=11)	Not Successful (N=21)	uOR (95% CI)	P
	N (%)					
Treatment category	Salvage	21 (66)	6 (29)	15 (71)	1.00	0.34
	Switch	11 (34)	5 (45)	6 (55)	2.1 (0.5-9.5)	
Geographical Region	Southern	4 (13)	2 (50)	2 (50)	1.00	0.97
	Central Western	7 (22)	2 (29)	5 (71)	0.4 (0.-3-5.2)	
	Northern	4 (13)	1 (25)	3 (75)	0.33 (0.02-6.66)	
	Central Eastern	16 (50)	6 (38)	10 (63)	0.60 (0.06-5.45)	
	Eastern	1 (3)	0	1 (100)	Not estimable	
Time in relation to failure	Before	3 (9)	1 (9)	2 (10)	1.00	1.00
	During	23 (72)	8 (73)	15 (71)	0.94 (0.1-6.3)	
	After	6 (19)	2 (18)	4 (19)	0.94 (0.07-12.0)	
	Median (IQR)					
RNA at failure	Copies/ml	1,436 (467-23,250)	23,000 (1,330-33,993)	773 (382-4960)	2.2 (0.99-4.9)	0.05
RNA at sample	Copies/ml	1,530 (367-15,986)	17,198 (2,517-59,100)	690 (270-1,790)	3.7 (1.4-10.0)	0.009
Time between failure and sample	Months	1.6 (0-3.1)	1.5 (0-3.0)	1.9 (0-4.6)	0.95 (0.84-1.08)	0.44
Time between RNA and sample	Days	0 (-13-0)	0 (-7-0)	0 (-35-0)	0.99 (0.96-1.01)	0.37
Date of sample	Year	2010 (2009-2011)	2010 (2010-2011)	2010 (2009-2011)	1.01 (0.60-1.71)	0.96

The total coverage and ratio of forward and reverse reads can be seen in Table 4.19 below, and showed good coverage and balance for those samples with successful genotyping.

1. Not applicable as only one read direction was reported.

Table 4.19. Coverage and coverage ratios for successfully genotyped samples.

Patient	Genotyping quality	
	Total Coverage Median (IQR)	Ratio FW/RV coverage Median (IQR)
2	2,877 (N/A[1])	1.00 (N/A[1])
5	8,092 (7,215-9,160)	1.00 (1.00-1.00)
11	10,500 (8,207-10,584)	1.01 (1.01-1.60)
13	1,370 (1,206.-2,491)	1.00 (0.98-1.28)
15	5,884 (4,201-6,900)	1.39 (1.0-1.62)
17	3,174 (2,560-3,918)	1.00 (1.00-1.00)
19	7,565 (7,532-8,322)	0.99 (0.65-1.00)
21	4,599 (3,755-5,552)	1.00 (0.80-1.00)
24	1,640 (1,527-1,825)	1.00 (1.00-1.01)
26	10,980 (10,647-11,405)	1.00 (0.88-1.00)
29	6,324 (5,222-11,837)	1.00 (0.99-1.00)

Resistance patterns at failure and baseline for all individuals who were successfully genotyped are shown in Table 4.20. Overall, 8/11 (73%) of individuals had any detected resistance upon VF and 4/11 (36%) had any detected integrase resistance. INSTI resistance patterns consistent with the N155H and Q148H pathway were detected, although the Y143H mutation was not detected. One individual (labelled as #13) developed a major accessory INSTI mutation, T97A, which can confer resistance to ELV. Individual #15 developed the mutations Q148H and G140S, which confer resistance to both RAL and ELV and at least partial resistance to DTG when administered using twice-daily dosing. Either of the mutations in isolation would confer resistance to DTG if administered using once-daily dosing. Individual #17 developed the N155H mutation which confers resistance to RAL, ELV and which could, depending on the dosing, confer resistance to DTG. A minority variant, E92Q was also detected in this individual, and when present in combination these two mutations confer resistance to DTG when administered using twice-daily dosingThe final individual with INSTI resistance, #29, had only the N155H mutation detected in the integrase gene. The N155H does confer resistance to DTG when this drug is administered once-daily. Although numbers were too limited to draw wider conclusions, this means that DTG susceptibility was likely to be compromised in at least 2 of 4 individuals experiencing RAL failure with detected INSTI resistance

Table 4.20 Resistance patterns among successfully genotyped patients

Patient	Baseline Resistance			Resistance at failure					Predicted INSTI GSS
	NRTI	NNRTI	PI	NRTI	NNRTI	PI	INSTI	New mutations	
2	*UN*[2]	*UN*	*UN*			I62V		*N/A*	RAL=1 EVG=1 DTG=1
5	M184V					L89M, I62V V77I, M36I		4	RAL=1 EVG=1 DTG=1
11	K219E, M184V K70R		L90M, A71T			M36I*, I93L L90M*,I62V L63P		4	RAL=1 EVG=1 DTG=1
13	*UN*	*UN*	*UN*	M184V	P225H, K103N	I93L*,M36I H69K,L89M	T97A	*N/A*	RAL=1 EVG=0 DTG=1
15	T215Y, L210W M184V, L74V, D67N, M41L	K103N, K101P	L90M, V82F G73S, A71V I62V, I54V M46I, K20R L10I	K70E,T215Y D67N,M184V* L210W, M41L	K103N	L33F,I54V V77I,I62V A71V,V32I L63P,K20R M46I, M36I K43T,L90M I93L, V82L I84V	Q148H, G140S	3	RAL=0 EVG=0 DTG=0.5

17	*UN*	*UN*	*UN*	F116Y,F77L A62V,M184V Q151M		M36I,I64V	N155H,E92Q*	*N/A*	RAL=0 EVG=0 DTG=0
19	M184V			L74V*		L90M, L63P A71V*, I93L		5	RAL=1 EVG=1 DTG=1
21	*UN*	*UN*	*UN*			I62V, L90M L63P, A71T I93L*		*N/A*	RAL=1 EVG=1 DTG=1
24	*UN*	*UN*	*UN*			I93L, M36I L63P,H69K		*N/A*	RAL=1 EVG=1 DTG=1
26	M184V	Y181C, V106A, V106I	L90M, L89V I84V, V82T A71T, I62V I54V, M36I K20M, V11I L10I		K103N*, V90I*	I62V,F53L L90M,K20M M36I*,V82A L63P,A71V			RAL=1 EVG=1 DTG=1
29	T215Y, L210W M184V, Q151M, F77L, V75I L74V, A62V, M41L	H221Y, G190A Y181C, K103N, A98G	L90M, L89V I84V, V82T, A71V, I62V, I54V, M36I K20M	M41L, T215Y, H221Y, L210W	V90I, K103N, Y181C,	V82A,L24I I64V,I54V L63P	N155H		RAL=0 EVG=0 DTG=1[2]

**describes minority variant, present in less than 25% of the circulating virus*

1. Excludes minor PI mutations; 2. UN=undetectable

2. Assumes twice-daily dosing of DTG; 0 if once-daily dosing.

4.5.Discussion

4.5.1. Time to and risk factors for RAL VF

In this analysis I found a probability of RAL failure of up to 12% by 7 years for individuals starting RAL with a suppressed bVL and 25% for those starting RAL with a raised bVL. Results from both ITT and PP analyses were very similar, although as expected rates were slightly lower in the PP analyses. Although direct comparisons between different studies should be done with caution, these findings are in line with those from both clinical trials (522–524,569) and observational studies (547,550,551,553,556) which have shown RAL to have durable virological efficacy among treatment experienced patients. However, the long-term estimates of risk of VF found here are lower than those reported in the BENCHMRK trial, where the 240 week KM estimate of time to virological suppression was 51% (524). This could reflect the fact that BENCHMRK required individuals to have experienced triple class failure in order for them to be enrolled. In the current study population the baseline GSS was relatively high and only 16.4% of individuals with available resistance data were found to have resistance to all three major drug classes.

The risk of VF differed according to baseline VL, and in agreement with findings from other authors was found to be considerably higher among patients starting RAL with a raised bVL (546,559–561). I chose to describe the results and risk factors separately according to the bVL, hypothesising that this would serve as a proxy for reason of RAL start. The reasons and risk factors for VF are likely to differ among people who switch to RAL for toxicity reasons and those who switch to suppress VL (559,560). However, as EuroSIDA does not collect the reason for starting a particular drug, I was not able to directly separate out these two groups and was forced to use bVL as a proxy for reason of starting RAL. To give an indication of how well splitting the study population according to bVL reflected the reason for starting RAL I looked at the reasons for stopping a drug in the month before RAL was started. Physician reported treatment failure was the most common reason for stopping a drug prior to starting RAL in the group with raised bVL, whereas toxicity (any kind) was the most common reason for stopping a drug prior to starting RAL in the group with suppressed VL, indicating that use of bVL was a reasonable proxy for reason of starting RAL.

I could not find any strong associations between the potential risk factors investigated here and the risk of experiencing VF among those starting RAL with a suppressed bVL. There were weak associations suggesting that the risk of VF decreased with higher baseline CD4 counts and more recent calendar year of RAL initiation (p=0.04 and 0.08 respectively). The fact that rates of

virological failure are lower in recent years have been consistently shown in cohort studies of people receiving cART with or without RAL (570,571). The associations with risk of failure was attenuated in the PP analysis as compared to the ITT analysis. Although PP analyses are often performed to evaluate the virological potency of a drug after removing the confounding effect of stopping due to toxicity, reduced statistical power is the most likely explanation of this finding.

In contrast, a number of factors were associated with the risk of experiencing VF among individuals starting RAL with a raised bVL. In terms of demographic predictors, individuals of a non-white ethnicity were at a higher risk of experiencing VF in multivariable ITT analyses. Individuals of a non-white ethnicity have been reported as having a higher risk of experiencing VF in a number of studies (572–574), and my findings are in agreement with this. However, the increased risk of VF was not as marked in PP analyses. Again, this could be due reduced statistical power or to the fact that genuinely the virological potency of RAL is the same regardless of ethnicity and the difference in the ITT analysis is due the difference in rate of failure in those who stopped RAL because of toxicity (excluded in the PP analysis). There is evidence that adherence patterns to ART differ by ethnicity (574) so it is conceivable that the Caucasian population was generally more adherent both to the RAL-based and subsequent regimens used and consequently at lower risk of VF.

The risk of VF was also significantly higher among individuals receiving care in Central East and Eastern Europe compared to Southern Europe. This is consistent with the results from previous EuroSIDA analyses (575–579), and there are a number of possible reasons that could explain these findings. RAL was used relatively rarely in Central Eastern and Eastern Europe compared to other European regions. This likely reflects the high cost of the drug (580), and could indicate that RAL is being prioritised for those individuals most in need or issues with the supply chain of RAL in these countries. This would mean that individuals in Central Eastern and Eastern Europe who receive RAL may be at a more advanced disease stage than those who receive it in other areas of Europe. Although my analysis took into account a number of factors associated with disease severity, including baseline CD4 count, CD4 nadir and treatment history, it is possible that differences in disease severity at time of starting RAL in East and West Europe are not fully captured by these measured indicators. However, it is also possible that the increased risk of VF in East and Central Eastern Europe reflect differences in the health care policy, as post-Soviet states have struggled to modernize an underfinanced and neglected healthcare system (581).

I identified a number of other risk factors for VF on RAL among those starting it with a raised bVL. There was weak evidence that individuals with higher baseline VL levels and lower nadir CD4 were more likely to experience RAL failure, in agreement with a study by Wittkop et al of the French ANRS cohort (556). Baseline VL was the factor most commonly identified as associated with an increased risk of VF in other analyses (543,550,551,556). The evidence supporting a difference according to levels of bVL among those starting RAL with a raised bVL was relatively weak in this analysis, something which could be explained by the fact that the the relatively small range of VL levels among those with a VL>200 copies/mL. It is possible that a pooled analysis, considering risk factors for those starting RAL with a raised and suppressed bVL together, would have shown that bVL was a stronger risk factor. However, the interpretation of such an analysis would be less clear-cut given the combination of two study populations for which the indication for starting RAL was different.

The raised risk of VF among individuals starting RAL with a raised bVL, as well as the weak linear association with bVL and the risk of VF identified here indicates that individuals with very high VL values who switch treatment in order to suppress VL are at a particularly high risk of experiencing VF. It is possible that a very high VL indicates a problem with adherence, which has previously been shown to be a key determinant of the success of RAL treatment (534). Although the role of adherence could not be evaluated specifically in this study due to a lack of availability of data, the results supports the recommendation that individuals with high bVL should be particularly targeted for adherence interventions. I did not find any differences in the risk of VF among individuals starting RAL with a raised bVL according to an individuals' exposure to mono or dual therapy before the use of cART or GSS of the RAL regimen, in contrast to Capetti et al who found that individuals with a more complex treatment history were more likely to experience VF (549). It could be that exposure to mono or dual therapy before starting cART is not an adequate measure of treatment history, and that the relatively high GSS of the regimens used in this study masked any negative effect.

Changing the viral load cut-off point, censoring individuals during times of infrequent VL measurements or using a more specific measure to define the bVL did not impact any of these conclusions markedly, suggesting that my findings were robust to the changes in the definitions used.

4.5.2. Resistance profiles following VF

The VL at failure among both patient groups was relatively low, and the availability of genotypic data limited. No individuals had reported data on integrase resistance testing done in clinics, and only 33 samples could be identified that met the criteria for genotyping. Of these 33 samples, 11 (34%) were successfully amplified and genotyped. Two factors acted as a strong determinant of genotyping success: VL at the time of failure and VL at the time of the sample. This indicates that the cut-off limits used to send samples for genotyping was too low in this study. The rationale for using such a low VL was based both on previous reports of successful genotyping at low VLs (566), and to ensure that the highest possible number of test results was available for analysis even if this meant a potentially large number of unsuccessfully genotyped samples.

Despite the limited data availability, the results from the genotyping showed some interesting results. Firstly, major RAL mutations were detected in 4/11 (36%) patients that were genotyped. The N155H mutation, which can compromise the response to DTG depending on the dosage and whether or not it is present with other mutations, such as Q148R or E92Q, was observed in 2/4 patients with INSTI resistance. The other resistance patterns observed were the Q148H/G140S pathway and a single instance of the accessory T97A, which primarily compromises the efficacy of ELV. Although minority variants were commonly detected in the RT and PR gene, only one minority variant in the integrase gene was detected: E92Q. This mutation occurred in combination with N155H, and when these two mutations are present in combination they confer resistance to DTG, also when it is administered twice daily (394). The pathways described here confirm findings from other analyses of INSTI resistance patterns (550,556).

4.5.3. Strengths and limitations

This analysis has a number of significant limitations. The first main limitation is the limited sample size. Although EuroSIDA is a large cohort with a reasonable number of individuals receiving RAL, the availability of resistance data and stored plasma samples was limited. I used a reasonably broad cut-off for identifying stored plasma samples of +/- 3 months if a sample during RAL failure was not available. This allowed me to capture samples that may give information on RAL resistance despite the potential for small inaccuracies in the dates collected. Resistance patterns on RAL can evolve rapidly, and following cessation of RAL resistance mutations can disappear as fast as 4 weeks after RAL withdrawal (582), indicating that a longer time-window would have risked sending samples for genotyping which would have inaccurately shown an absence of INSTI resistance. A second limitation is the relatively low success rate of the genotyping (36%). This seemed to primarily be explained by the

relatively low VL levels at the time of the sample. Although other studies have demonstrated that genotyping can be done at low VL levels (389,566), technology, operating protocols and conditions differ across different laboratories. In order to ensure efficient use of EuroSIDA funding, future studies utilising the EuroSIDA plasma bank to genotype individuals should consider prioritising samples taken during periods of higher viral replication. Nonetheless, the number of individuals genotyped for resistance (N=11) is still as high or higher than a number of previous studies (534,545,551,553,556,566) and the amount of information provided by the NGS platform is very valuable and not available in many other settings. A further limitation is the restriction of this chapter to only studying RAL use, and not EVG and DTG. This was mainly due to the low number of people with exposure to these drugs in EuroSIDA at the time of this analysis.

In terms of the analysis of virological efficacy, a limitation is the lack of a control group to put the failure rates into context. This was a conscious decision, which was taken given the difficulties in identifying a reasonable comparison group using routinely collected observational data. Another limitation of using observational data is the potential variation in and infrequency of VL measurements, although the sensitivity analysis where individuals were censored 6 months after each measurement led to consistent results. The final limitation is the potential lack of generalizability. The individuals with data available for inclusion in this analysis differed from those that could not be included, and the conclusions are not likely to be generalizable to all individuals living with HIV in Europe. These limitations are characteristic of most observational studies, and have to be balanced with the benefit of analysing data from routine clinical care, such as generalisability that is likely greater than that in clinical trials which employ much stricter inclusion criteria.

4.5.4. Conclusion

This analysis adds to a growing body of evidence showing that RAL has durable virological efficacy over long periods of FU, in this case, of up to 7 years with a maximum of 25% experiencing virological failure by this time on the drug if started with a VL >200 copies/mL. The risk of VF was higher among individuals who started RAL with a higher baseline viral load, and particular attention should be given to these patients in order to ensure that they promptly achieve viral suppression. Other risk factors for VF on RAL among individuals starting the drug with a raised bVL include a low CD4 nadir, geographical region and possibly non-white ethnicity. Very few individuals had data available on INSTI resistance, even after central genotyping of samples. Nonetheless, I identified a number of mutations following RAL failure that can compromise the efficacy to DTG. Although DTG has shown reasonable efficacy among RAL experienced patients in clinical trials, these findings suggest that sequential use of RAL and DTG should be accompanied by resistance testing. The role of minority INSTI mutations on consequent treatment responses to DTG requires further research, and existing cohort studies should prioritise the collection of high-quality information on integrase resistance in order to provide data on the evolution of INSTI mutations in routine clinical practice. More detailed suggestions for future research are provided in Chapter 8.

4.6. Dissemination of results

An early version of these results was presented at the 2015 Antiviral Therapy Meeting in London and the 2015 European AIDS conference (Appendix VII). A manuscript is in preparation.

Chapter 5 . The effect of drug resistance mutations on CD4 cell decline in HIV positive individuals maintained on a failing treatment regimen

5.1. Introduction and Objectives

In Chapter 3 and 4 I showed how virological failure with resistance is becoming increasingly rare, particularly in high income settings such as Western Europe. However, this situation is not homogenous across geographical regions. As many as 17% of individuals starting first line regimens in South Africa may experience virological failure (VF) within 5 years of starting treatment (583), and a recent systematic review also suggests that individuals in low income settings may be more likely to have resistance once they are diagnosed with VF compared to individuals in high income settings (584). This is likely due to how people are monitored,how quickly treatment is modified as well as the number of available treatment options in low income settings as compared to high income settings. Although an effective second or third line regimen of antiretrovirals not previously used, including those from new drug classes, can often be prescribed for individuals who experience VF (585), constructing a suppressive regimen can still be challenging for people whose viruses have complex resistance patterns or because of limited drug availability. This is particularly the case in low income settings, where access to 3rd or higher line regimens is generally limited (586). This could affect a potentially large number of people. In a 2010 multicohort analysis, Pujades-Rodriguez and colleagues estimated that 19% of individuals receiving second line therapy in a variety of low income settings met WHO failure criteria after a median of 11.9 months following the start of second line therapy, meaning they would need access to third line regimens (587). In situations such as these, people living with HIV may be forced to take a non-suppressive regimen for extended periods of time (72,588).

It has been known for over a decade that individuals who receive a non-suppressive ART regimen can still derive a clinical benefit from the treatment and may experience stable or increasing CD4 counts despite ongoing viral replication (332,364). In a small 2001 trial of 23 men, Deeks et al found that people who were maintained on an ART regimen despite having a detectable viral load had higher CD4 counts and lower viral load levels over time compared to those who interrupted treatment (332). This was proposed to be due to a combination of

residual antiretroviral activity and the preservation of a virus population with low replicative capacity (RC). In 2004, the large PLATO collaboration reported that among individuals experiencing triple-class failure, CD4 counts increased as long as viral load levels were below 10,000 copies/ml, and that those receiving more drugs experienced more favourable CD4 count changes despite ongoing VF (364). The authors suggested that this latter result could be due to more drugs leading to more resistance mutations and thus a lower RC. Several additional studies have also shown stable or increasing CD4 counts despite the presence of ongoing viral replication (589–591), and a low RC has been directly linked with beneficial CD4 count changes (592–594).

Although there is agreement that the composition of a regimen that fully supresses viral replication should be the aim of cART (507), this is not always possible. It has been suggested that the preservation of certain resistance mutations that are known to reduce RC may clinically benefit individuals for whom a fully suppressive regimen cannot be constructed. Specifically, maintaining patients on lamivudine (3TC) in order to preserve the M184V mutation that has been repeatedly associated with lower fitness levels (595,596) has been proposed as a potential treatment strategy (597). However, the evidence available in support of this strategy is far from strong. A small proof-of-concept study by Castagna et al evaluated this hypothesis, and they found that patients remaining on 3TC monotherapy experienced improved immunological and clinical outcomes compared to patients who interrupted all treatment (598). However, the COLATE randomized controlled trial found no added immunological or virological benefit of maintaining patients on Lamivudine in the context of cART after patients had experienced VF (599). The COLATE investigators suggested that any effects of a M184V preserving strategy may have been masked by the effect of potent cART, as most individuals in the trial switched to a new regimen with a high genotypic sensitivity score (GSS). It is possible that any benefits of an M184V preservation strategy may only be seen for patients with few or no remaining treatment options. Despite this limited evidence in support of a fitness targeting treatment strategy, 2016 updates of HIV treatment guidelines still mention M184V preservation strategies as a potential treatment approach for special cases where treatment options are severely limited (600,601). The role of other mutations on CD4 count trends in the context of VF also remains largely unknown, and whether the effect of a reduced RC on the CD4 count occurs via viral load replication (e.g. HIV-RNA levels) or through other mechanisms has also never been convincingly determined.

Given this lack of evidence, I used data from the EuroSIDA cohort, the UK CHIC cohort and the UK HIV Drug Resistance Database (RDB) to evaluate the effect of drug resistance mutations (DRM) on CD4 count changes among patients who are maintained on a failing treatment regimen. The specific objectives for the analyses were as follows:

1) Estimate differences in CD4 decline among individuals with and without drug resistance who remain on a failing treatment regimen.

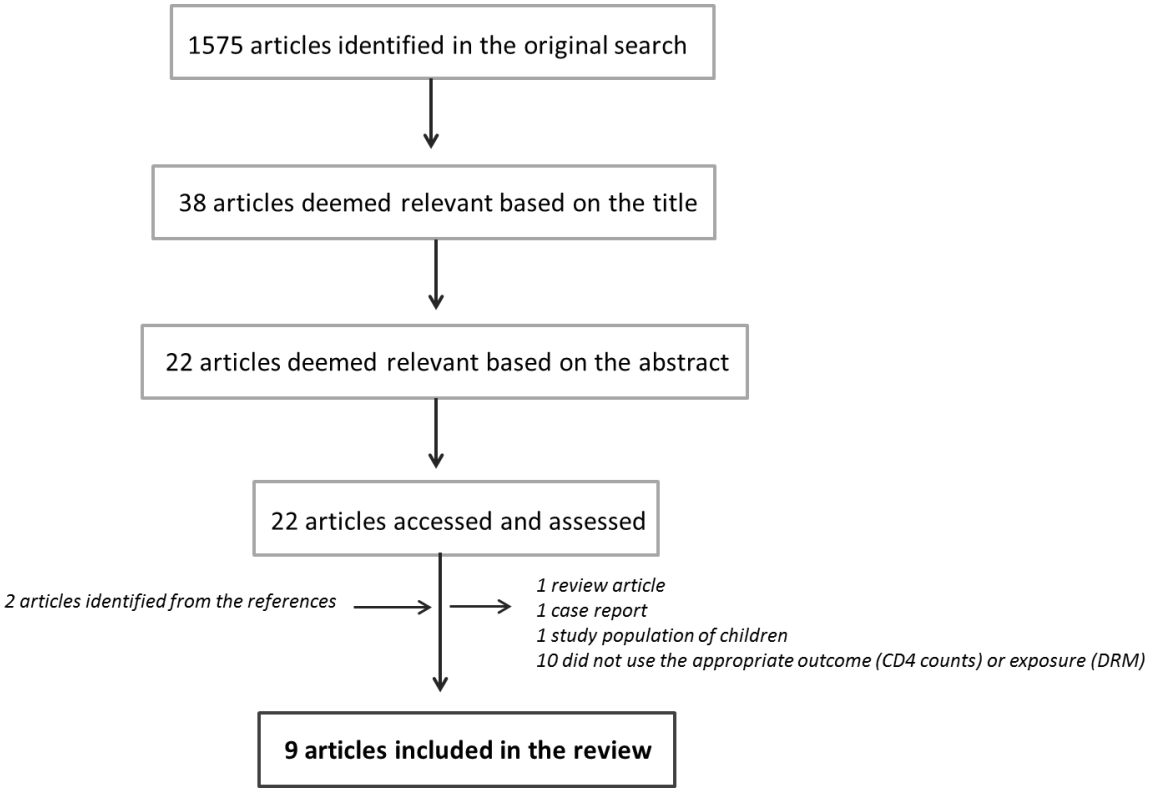
2) Evaluate the effect of detected resistance to either of three major drug classes (NRTI, NNRTI and PI) and individual DRM on CD4 decline among individuals with at least one DRM detected.

3) Identify clusters of DRM using principal component analysis (PCA) and investigate the effect of these clusters on CD4 decline.

5.2. Literature Review

I conducted a literature review with the aim of investigating what was known about the effect of DRM on CD4 count changes. The initial search strategy (Chapter 2, page 74) returned 1,565 articles, of which 38 were deemed potentially relevant based on their title. This was reduced to 22 articles after abstract-scanning, and these 22 papers were assessed for inclusion in the review. The majority of these were excluded as they used direct laboratory measures of the replicative capacity as either the exposure or outcome rather than DRM and CD4 counts specifically (N=10). As their findings are nonetheless relevant to the topic, these are summarised where relevant in the discussion. In total, nine articles addressed the specific question of the effect of resistance on CD4 counts and were therefore included in the review (Figure 5.1).

Figure 5.1. Identification of articles for inclusion in the literature review



A summary of the articles that formed part of the review can be found in Table 5.1. Briefly, the majority of the studies (5/9) were small, with fewer than 100 participants, and one was a mathematical model based on simulations rather than real data. Despite this, there were some interesting findings. Three studies found evidence that the detection of individual mutations were associated with CD4 count levels. De Mendoza et al performed a relatively large cross-sectional analysis of 825 individuals living with HIV in Spain who had a GRT done at some point between 1999 and 2002 (602). In univariable analyses, the presence of any DRM, the K70R and M184V mutation were associated with higher CD4 counts, whereas presence of the L74V and the number of both primary and compensatory PR mutations were associated with lower CD4 counts. After adjusting for viral load, only the number of compensatory PI resistance mutations and detection of K70R remained associated with CD4 counts. The detection of any drug resistance did not seem to correlate with CD4 counts.

Although this study indicated that patients may gain some absolute CD4 count benefit from remaining on a regimen to which the virus has developed resistance, it suffered from a number of weaknesses. First of all, the study population consisted of both treatment experienced and treatment naive individuals (although the number of treatment naive individuals was relatively low). Secondly, results which were not significant in the multivariable results (i.e. those with an associated $p>0.05$) analysis are not presented, which makes it difficult to assess whether the odds ratios shifted towards one following adjustment, or whether the analysis simply did not have high enough power to detect a significant difference. And finally, as this was a cross-sectional analysis it is hard to draw any conclusions regarding the causal effect of DRM on CD4 counts, and the study provides no evidence to support that DRM may be associated with CD4 changes over time.

Nicastri et al reported results from a cohort of 43 individuals who received care at two clinical sites in Italy (603). Despite the small sample size, the authors found that presence of M184V was associated with a viroimmunologically discordant response to HAART, defined as having a CD4 count increase of 100 cells/mm^3 or more despite ongoing VF, after controlling for a number of potential confounding factors (OR=25.48, 95%CI=1.43-453.93). The final study, by Antinori et al, also took place in Italy and reported data from 354 patients who had a genotypic resistance test result available and were experienced VF at this time (604). In this cross-sectional analysis, the odds of discordant virological responses, defined as above, were found to be higher among individuals with the RT M184V (OR=1.92, 95%CI=1.25-3.07) mutation or the L241 (OR=6.18, 95%CI=1.77-21.55) and V82A (OR=1.75, 95%CI=1.06-2.89) mutations. The

Y181C mutation was associated with lower odds of CD4 count recovery (OR=0.35, 95%CI=0.18-0.65). After adjustment for a range of factors, including VL, the L24I and Y181C associations remained statistically significant. Similar to the analysis by De Mendoza et al, insignificant multivariable results were not present in the paper, which makes it hard to assess whether the attenuation of the association with the M184V and V82A mutations was an issue related to power, or whether the effect of these mutations on CD4 counts were at least in part mediated through viral load.

In contrast, five studies found no association between resistance measured in a number of different ways and CD4 counts, including a large and relatively recently published study longitudinal study of 826 patients from the Italian ARCA cohort by Gianotti et al from 2011. However, as the hypothesis behind this analysis was that DRM would interfere with immunological recovery, its design allowed individuals to switch regimens. Given that mutations can disappear from the majority circulating virus relatively quickly following the cessation of selective drug pressure (326), it is possible that this design feature masked any potential associations. The odds ratios for M184V in the multivariable model also indicated that the detection of M184V was possibly associated with discordant immune-virological responses, but that the study may not have had sufficient power to detect this (OR=1.31, 95%CI=0.97–1.77,p=0.076). The mathematical modelling study by Vaidya et al suggested that the presence of Enfuvirtide resistance could result in beneficial CD4 counts despite ongoing viral replication among individuals who are re-administered this specific drug following VF on it, but as this analysis was not based on clinical data the results are somewhat speculative. As the EuroSIDA resistance database does not contain genotypic data of HIV regions outside RT and PR this hypothesis could not be directly tested in this chapter.

5.2.1.What this analysis adds

Previous studies have been relatively small and have found conflicting results regarding the impact of DRM on CD4 count changes. Many have only presented partial results, meaning that adjusted estimates of the effect key mutations such as M184V on CD4 count changes have not been published. The studies have also used a range of inclusion criteria, many have been cross-sectional and most assessed CD4 response using a binary immunological discordance categorisation. Using the EuroSIDA cohort and UK CHIC/UK HIV RDB presents an opportunity to evaluate the association between detection of DRM and CD4 count changes as these large cohorts hold historical data on a population which may have been exposed to failing regimens for relatively long periods of time. In addition, the large size of both contributing studies

allowed me to derive more accurate estimates of the effect of DRM on CD4 counts over time among patients who remain on failing regimens compared to currently available estimates.

Table 5.1. Papers reporting on the effect of drug resistance on CD4 counts following VF, by year of publication					
Author	**Year**	**Design and Setting**	**Study Size**	**Main Results**	**REF**
Gianotti et al	2011	Longitudinal analysis of individuals who had a GRT at VF (VL>50 while on ART) between 2000-2004 in the Italian ARCA cohort	826	No individual mutations in the pol gene were associated with an increase or decrease in the odds of immune recovery, defined as an estimated CD4 slope greater than zero, after adjustment for potentially confounding factors including VL (defined as the viraemia detectability ratio). However, a number of mutations were associated with the odds of immune recovery in univariable analysis. These included 154V (OR=1.60,95%CI=1.12–2.29,p=0.009) and V82A (1.63, 95%CI=1.14–2.33, p=0.007). M184V was only borderline associated with the odds of immune recovery in both univariable (OR=1.26, 95%CI=0.96–1.66),p=0.097) and multivariable analyses (OR=1.31, 95%CI=0.97-1.77, p=0.08).	(605)
Vaidya et al	2010	Mathematical model of the effect of Enfuvirtide (ENF) use despite VF and extensive drug resistance	N/A	Results from the modelling suggests that re-administration of ENF following VF and resistance development to the drug had no impact on VL levels, but still allowed for CD4 count increases due to the lower fitness of the ENF resistant virus.	(606)
Solomon et al	2003	Laboratory study of CD4 cell activation, CD4/VL responses and viral genotype in HIV outpatient clinics in Melbourne	81	The authors found no association between any specific or the total number of mutations and discordant immunological/virological response, defined as confirmed VL>400 and a CD4 slope above 0, although presence of the Y181C mutation was associated with non-response to therapy, defined as a CD4 slope below 0 and a confirmed VL >400. Specific effect sizes were not reported in the analysis.	(607)
De Mendoza et al	2005	Cross-sectional analysis of patient data (both treatment naïve and those undergoing VF, as defined by treating physicians) submitted to a Spanish reference laboratory between 1999 and 2002	825 (762 treatment experienced patients).	Patients with DRM had higher median CD4 counts in univariable analyses, but not after adjusting for VL. After VL adjustment, the number of compensatory PI resistance mutations were associated with lower CD4 counts (-15.39 cells/mm^3, 95%CI=23.32-7.47, p <0.001). K70R and M184V were associated with higher and L74V with lower CD4 counts, but only K70R remained significant after VL adjustment (+48.78 cells/mm^3, 95%CI=3.72-93.82, p=0.034).	(602)
Nicastri et al	2003	Analysis of the genotypic and virological profiles among patients with immunologically discordant responses, defined as a confirmed VL>3000 but increasing CD4 counts (>100 cells/ml more compared to pre-HAART values)	43	Only the M184V mutation was significantly related to experiencing discordant immunological virological responses (OR= 25.48, 95%C=1.43 -453.93)	(603)

Dionisio et al	2003	Analysis of patients experiencing VF (defined as a rebound after initial suppression to undetectable levels, or a decline in HIV RNA of <0.5 or 1.0 log by 4 and 8 weeks of treatment) at two outpatients clinic in Italy during a 3 year period.	16	The total number of resistance mutations was correlated with the RNA slope (r=0.554, P=0.026) but not the CD4 slope (r=–0.251, p=0.348).	(608)
Barbour et al	2002	Longitudinal study of adults on a stable PI based regimen despite ongoing and persistent viral replication (HIV RNA>500 copies/mL)	20	No difference in the resistance phenotype was found between patients who experienced CD4 count declines during prolonged failure and those who did not. Effect estimates were not provided.	(609)
Antinori et al	2001	Longitudinal study of individuals experiencing VF (defined as a reduction of the HIV RNA of less than 1 log after 8 weeks or persistent HV RNA >80 copies/mL after 6 months of treatment or any rebound, confirmed with at least two consecutive tests after reaching undetectable viraemia) with a GRT from two outpatient centres in Rome	354	The presence of M184V, L24I and V82A was associated with higher odds immunological recovery (defined as an increase in the CD4 cell count of greater than 100 cells/mL from starting HAART until the GRT), whereas Y181C was associated with lower odds of immunological recovery in univariable analyses. After adjustment, only the L24I (OR=6.93, 95%CI=1.80-26.57,p<0.01) and Y181C (OR=0.40, 95%CI=0.20=0.80, p<0.01) associations persisted.	(604)
Belec et al	2000	Cohort of individuals on PI-based cART comparing discordant immunologic and virologic responders (failure to achieve an undetectable HIV RNA but nonetheless experiencing an increase in the CD4 count of greater than 50 cells/L as compared to baseline at months 3,6,9 and 12) to both complete responders (suppressed VL and increased CD4 count) and non-responders (not suppressed and decreasing CD4 count).	59	The number of primary and secondary PI mutations was higher among individuals with discordant responses (primary mutations: 0.92 ± 0.14; secondary mutations: 1.85 ± 0.22) compared to complete responders primary mutations: 0.12 ± 12; secondary mutations: 1.62 ± 0.32, but was only slightly lower than among complete non-responders (primary mutations: 1.25 ± 0.16, secondary mutations: 2.88 ± 0.29). The pattern of mutations did not differ between discordant and non-responders.	(610)

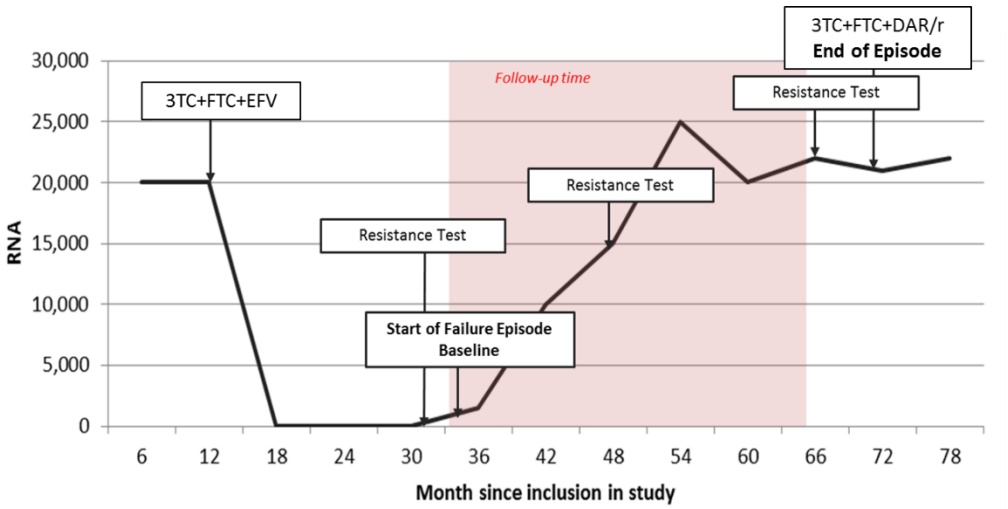
5.3.Methods

5.3.1. Inclusion criteria

For this chapter, I used the D40 update of the EuroSIDA database and the 2014 version of the UK CHIC cohort/HIVDRD. The datasets held data on 18,914 and 47,201 individuals respectively. For this analysis, I defined an episode of virological failure (VF) as 2 or more consecutive measures of VL >500 copies/mL over any time frame while the individual was kept on exactly the same ART regimen. Duplicate VL measures were cleaned out before the start of the analysis using a standard algorithm developed by the EuroSIDA cohort. This involved removing any duplicate VL dates with discordant VL values were from the dataset. I only considered periods of time when individuals were kept on a failing regimen, as mutations may disappear relatively quickly once drug pressure is removed.

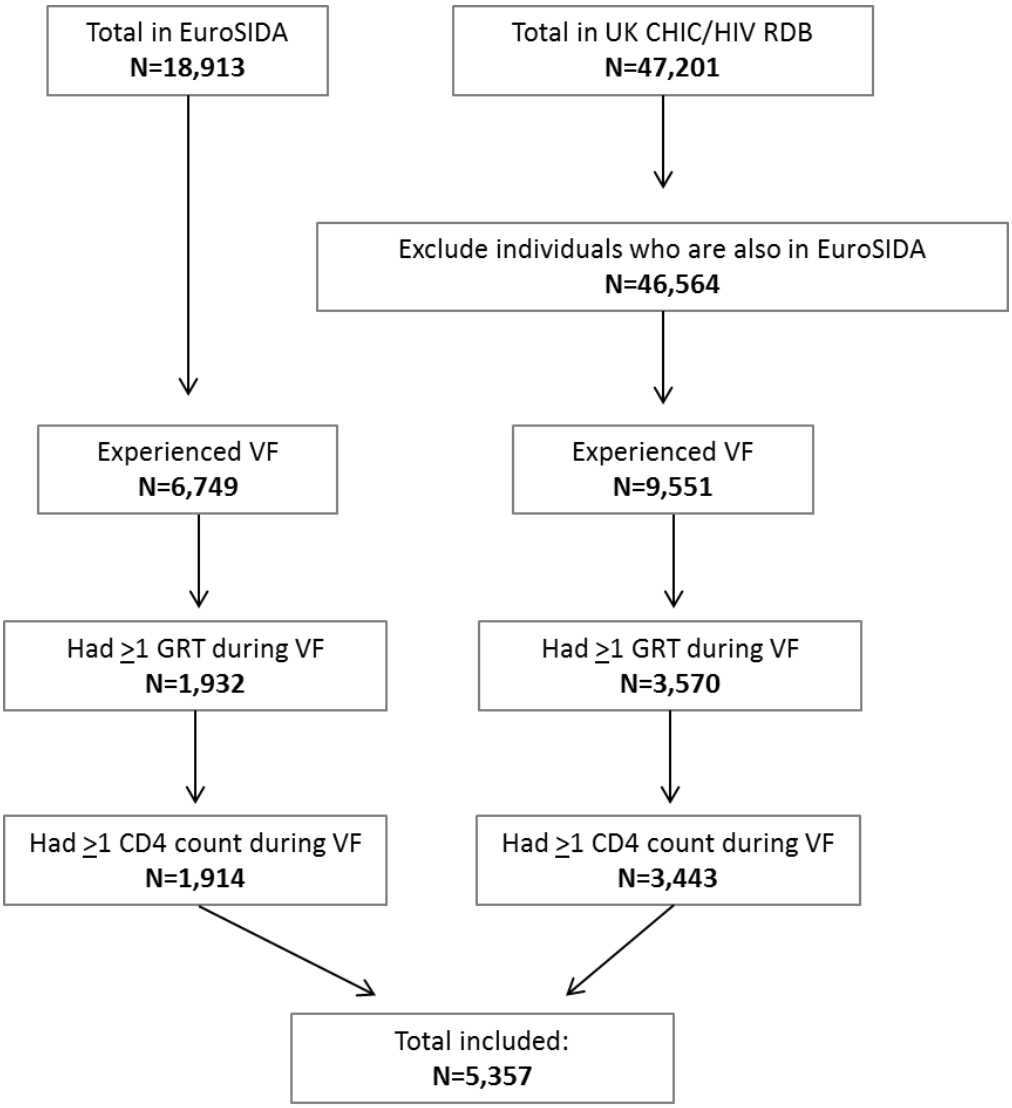
The start date of an episode was defined as the first viral load (VL) measurement >500 copies/mL, and the end date the first VL measurement below 500 or at the point where the ART regimen was changed (defined as either stopping or adding one or more drugs to the regimen). I required two consecutive high viral loads to ensure that we did not include individuals who were experiencing viral blips. The threshold of 500 copies/ml was chosen for two reasons: i) the episodes covered calendar times during which, because of the availability of the assays, viral load could not be detected below 500 copies/mL and ii) historically, it has been challenging to genotype at lower viral loads. During each failure episode, I further required individuals to have at least one resistance test and at least one CD4 count (611). An individual contributed all CD4 count measurements taken after the start and before the end of each failure episode to the analysis, and the baseline date for the estimation of the CD4 count slope was defined as the date of the first available CD4 measurement after the start date of the episode. Participants could contribute one or more failure episodes. A graphical illustration of how failure episodes were defined can be seen below in Figure 5.2. A list of patients in UK CHIC who are also in EuroSIDA was available from the UK CHIC statistician (*S. Jose, UCL*), which allowed me to ensure that duplicate patients were not included.

Figure 5.2. Hypothetical data illustrating the inclusion of failure episodes



Hypothetical Data. The start of a VF episode was defined as the first of a of >=2 consecutive VL measurement >500 copies/ml after at least 6 months of ART exposure. The end of a VF episode was defined as the date a change in the drug regimen occurred or when VL<500 copies/ml, whichever occurred first. An individual contributed all CD4 counts occurring at or after the start of failure until the end of the failure episode. Baseline was defined as the first CD4 count taken at or after the start of failure. Detected resistance was presumed to be present throughout the duration of the VF, irrespective of when it was detected.

Figure 5.3. Selection of individuals for inclusion in the study



5.3.2. Resistance data

In order to define antiretroviral drug associated resistance, I used all of the 4 major genotypic interpretation systems (IAS, Stanford, ANRS and Rega). These systems are based on slightly different mutation lists, and as I wanted to be able to study a large number of potentially important mutations I decided that it was too restrictive to use just one classification list. Although the Stanford, ANRS and Rega systems commonly attach scores to mutations, I only considered the presence or absence of each mutation for the construction of any and class-wide resistance categories. I used the IAS list to classify minor PI mutations, as this is the only drug resistance mutations algorithm that clearly distinguishes between minor and major mutations. Any resistance was defined as the detection of at least one mutation mentioned in either of the 4 classification systems. Individuals with only minor PI mutations were considered as having no detected resistance. The class-wide resistance categories studied were: NRTI resistance, NNRTI resistance, any PI resistance excluding minor PI mutations and any PI resistance including minor PI mutations. I evaluated the effect of all individual mutations that were detected in >1% of episodes, as mutations below this threshold were deemed too rare to be of potential clinical relevance. Resistance was presumed to be present from the start of the failure episode until the end, irrespective of at which point during the episode it was detected by the genotypic test. If there was more than one resistance test done during the same VF episode, all resistance tests contributed to the construction of the resistance exposures.

5.3.2.1. Principal Component Analysis

As mutations often co-occur in patterns that are particularly favourable for viral survival, we conducted a principal component analysis (PCA) to identify clusters of mutations. As explained in more detail in Chapter 2, PCA is a variable reduction technique that transforms a number of correlated variables into a smaller number of linearly uncorrelated variables, principal components (PC), that explain as much of the variance in the original dataset as possible (612). The contribution of each original variable to a given PC is given by a weight (loading), and these can be used to infer clusters of mutations that all load onto the same PC. Once PCs have been extracted, individuals in the dataset can be assigned a score on each PC which represents how closely their mutation pattern aligns with that captured by the PC. These scores can be used as exposures in consequent models (612). For this analysis I converted each PC from a continuous score into a binary exposure variable, using the 3rd quartile as a cut-off point.

I identified how many components to retain through graphical inspection of a scree plot, and retained components subjected to a varimax (orthogonal) rotation (451). Factor loadings below 30 were considered negligible, between 30-40 weak, above 40 strong and above 70 very

strong. Mutations with loadings above 40 were interpreted as forming part of a cluster captured by that PC; those with loadings between 30 and 40 were considered weakly associated with the cluster.

To simplify the interpretation of our results and because I expected that most clusters would be gene specific, I ran separate PCA's for RT and PI mutations. For simplicity and to aid the interpretability of my results, I did not differentiate between different amino-acid substitutions in the same position. The PCA was conducted using only the first VF episode for each individual, meaning that each individual contributed only one line of data.

5.3.3. Statistical methods

Characteristics of participants in the two cohorts and of individuals with and without resistance across the whole study population were compared using the basic statistical methods described in Chapter 2. I compared CD4 slopes using linear mixed models according to the presence or absence of (1) any resistance, (2) class of resistance and individual DRM and (3) according to the PCA derived scores. I did so using the PROC MIXED procedure in SAS (613). The study population varied according to the objectives of the analysis:

1) Among all individuals I compared the CD4 decline according to presence and absence of any resistance as defined in section 6.3.2.
2) Among those individuals with any resistance I compared the CD4 decline according to presence and absence of class-wide resistance and all individual DRM that reached the prevalence threshold. Individuals with no resistance were excluded from this analysis.
3) Among those individuals with any resistance I compared the CD4 decline according to those with "high" (>Q3) and "low" ($\leq$Q3) scores. Individuals with no resistance were also excluded from this analysis.

I restricted the study population for objective (2) and (3) as a raised VL levels without detected resistance may be indicative of ART non-adherence. Throughout the chapter I used a 2-level random effects model with a random intercept and a random slope for time. The two levels represented each episode and the individual CD4 count measurements. This allowed CD4 slopes slope (i.e. the change over time in the CD4 count value) and intercepts (i.e. the CD4 count value at the start of the viral failure episode) to vary between episodes, although the episodes themselves were presumed to be independent. I evaluated the use of a 3-level random effects model with an added level for individual, but this was found to explain a very small additional proportion of the variance and led to convergence problems when studying

the effect of individual DRM. In addition, the estimable results differed very little according to the inclusion further random effects. It was therefore decided to use a 2-level model. I used an unstructured covariance matrix for all models.

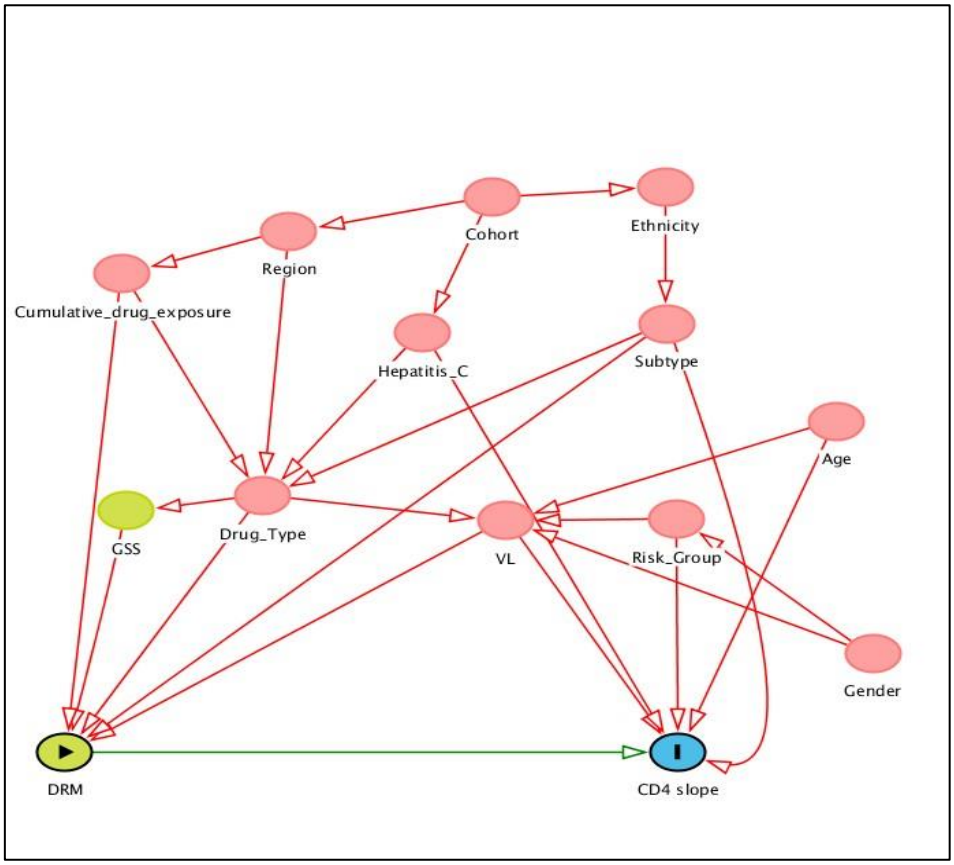
As I chose to test specific DRM on the basis of a prevalence threshold and not an *a-priori* hypothesis, I adjusted these p-values using the Benjamini-Hochberg procedure for correcting the false discovery rate (FDR) (614). As described in Chapter 2, this produces a q-value, which converts the p-value into a corrected value that represents the probability of a significant finding being a false positive. I used a q-value threshold of 0.05 to indicate significant results.

5.3.3.1. Model building strategy

Potential confounders were identified using guidance from clinical experts and visualised in a directed acyclic graph (DAG) as described in Chapter 2. The DAG showing the causal assumptions of this analysis can be seen in Figure 5.4. Assuming that this DAG completely describes the clinical situation, it implied that adjustment for age, hepatitis C status, risk group, subtype and viral load would allow me to estimate an unconfounded effect of DRM on CD4 counts. Although it was considered as a potential confounder in my DAG, VL can also be considered as a causal pathway variable, as the effect of any mutation on replicative capacity may at least in part be mediated through changes in the VL. For this reason, I conducted step-wise adjustments as follows:

1) Exposure alone, as defined in 5.3.3.
2) Model 1 + age, hepatitis C status, risk group and subtype
3) Model 2 + time-updated VL

Figure 5.4. DAG for the effect of DRM on CD4 decline



5.3.3.2. Variable categorisation

Individual mutations were entered in the models as binary variables, and separate models were constructed for each exposure variable. I modelled CD4 counts linearly on a raw (cells/mm^3) scale to ease the interpretation of the results. Viral load (log_{10} scale) was included on a continuous scale and age categorised into 4 roughly equal sized categories after checking linearity assumptions. The remaining covariates were categorised according to the principles laid out in Chapter 2 (Table 4.5).

Table 5.2 Covariates included in the model		
Variable	**Categories**	**Time-updated**
Hepatitis C co-infection[1]	Yes, No, Missing	No
Mode of transmission	MSM, IDU, Heterosexual, Other	No
Subtype	B, Non-B	No
Age(years)	35, 35-40, 40-45, >45	No
Viral Load (log copies/mL)	Linear, per log_{10} scale	Yes

1. *Defined as having a HCV antibody positive test at some point before baseline*
2. *Due to model convergence problems (infinite likelihood) experienced when including VL as linear on the log10 scale for three specific mutations, VL was included as a categorical covariate in the multivariable models (<1000, 1000-5000, 5000-10000, 10000-20000, 20000-100000, >100000). This is highlighted through footnotes in the results.*

I also estimated the level of viral suppression by comparing current VL levels with an individual's viral set point, which was defined as the mean of all pre-ART VL measures. Current VL measures equal to or above the viral set point were said to be 'Not supressed', measures less than 1.5 $\log_{10}$ copies/ml below the set point were said to be "somewhat supressed" and VL measures more than 1.5 $\log_{10}$ copies/ml below the set point were said to be "supressed". Unfortunately, the viral set point could only be calculated for a subset (N=2196) of people who had pre-ART VL data available. For this reason, it was not adjusted for in multivariable models.

5.3.3.3. Sensitivity analyses

I conducted sensitivity analyses in order to evaluate the reliability of some of our assumptions and analytical strategy. These were:

1) Excluding virological failure episodes during which the VL fluctuated greatly, defined as at least a doubling of the VL as measured on a $\log_{10}$ scale between any two VL measures taken during an episode (364), in order to exclude treatment of recent infections and large VL fluctuations potentially caused by poor adherence.

2) Requiring individuals to have at least 3 CD4 counts during the VF episode compared to at least one in the main analysis

3) Additionally, adjusting for the class of drug used (NNRTI, PI (single), PI (boosted) and Other)

5.4.Results

5.4.1. Characteristics of the study population

5,357 individuals contributing 7,661 failure episodes were included in the analyses; 2,757 (36%) from EuroSIDA and 4,904 (64%) from UK CHIC/HDRB. Individuals contributed a median of 3 (IQR=2-5) CD4 measurements during a median of 1 (Range=1-9) failure episodes that lasted for a median of 5 (IQR=2-13) months. Baseline characteristics of the participants can be seen in Table 5.4 and Table 5.3. Overall, the study population consisted primarily of white (68%) males (76%) who had acquired their HIV infection through sex with another man (55%). The median VL at the start of episodes was high (8,200, IQR=1,829-52,672 copies/ml] and the CD4 count relatively low (257, IQR=145-393, cells/mm^3). The majority of episodes also started with VL values that were lower than their estimated viral set point values (73.6% of those that had an estimated set point). The median GSS at the start of each episode was 1, meaning that on average participants were using only one drug which was predicted to be active against their virus at the beginning of the episode, and 25% of the study population was receiving a regimen that was estimated to have no activity (IQR=0-3).

There were some differences between individuals included from UK CHIC and from EuroSIDA (Table 5.3), most probably reflecting known differences between HIV positive people in the UK as opposed to mainland Europe. Individuals in EuroSIDA were more likely to report injecting drug use ($p<0.001$) and more likely to be infected with a subtype B virus ($p<0.001$). They also had lower RNA values at baseline (median: 7,200 vs 8,928 copies/ml, $p<0.001$). Individuals from EuroSIDA contributed double the FU time per episode than individuals from UK CHIC (median: 8 vs 4 months, $p<0.001$) and had a lower average GSS at the start of their episodes (median: 1 vs 2 active drugs, $p<0.001$), likely reflecting the fact that in EuroSIDA there is a higher proportion of patients entering the cohort with extensive previous use of antiretrovirals as compared to UK CHIC.

There were also some differences between those individuals who had resistance detected at baseline and those who did not (Table 5.4). The viral load was considerably higher during VF episodes where no resistance was detected ($p<0.001$), and, intriguingly, there was some suggestion that CD4 counts were also higher during episodes with no resistance, although this finding was only marginally significant ($p=0.05$). Episodes where no resistance was detected also tended to be shorter ($p<0.001$) and to have shorter gaps between CD4 measurements ($p<0.001$).

Table 5.3. Characteristics of the study population according to source cohort, per episode

		Total Episodes	EuroSIDA	CHIC	P-value
		N (%)	N (%)	N (%)	
At baseline, per individual		5357	1914	3443	
Gender	Male	4088 (76.3)	1501 (78.4)	2587 (75.1)	0.007
	Female	1269 (23.7)	413 (21.6)	856 (24.9)	
Ethnicity	White	3592 (68.2)	1590 (83.9)	2002 (59.4)	<.001
	Non-white	1673 (31.8)	306 (16.1)	1367 (40.6)	
Risk Group	MSM	2862 (55.0)	940 (51.8)	1922 (56.7)	<.001
	PWID	485 (9.3)	330 (18.2)	155 (4.6)	
	Heterosexual	1726 (33.1)	494 (27.2)	1232 (36.3)	
	Other	134 (2.6)	51 (2.8)	83 (2.4)	
Subtype	B	3659 (72.2)	1430 (87.9)	2229 (64.8)	<.001
	Non-B	1409 (27.8)	197 (12.1)	1212 (35.2)	
Hepatitis C	Yes	698 (9.1)	83 (9.7)	3891 (57.2)	<.001
	No	4461 (58.2)	569 (66.4)	615 (9.0)	
	Unknown	2502 (32.7)	205 (23.9)	2297 (33.8)	
Age (Median, IQR)	Years	38.5 (34.6, 42.9)	39.5 (34.8, 46.2)	38.2 (34.6, 41.8)	0.092
Baseline date (Median, IQR)	Year	12/00 (06/98-11/04)	11/98 (106/97-11/01)	07/02(05/99-03/06)	<.001
During FU, per episode		7661	2757	4904	
Viral suppression[2]	VL above set point	745 (26.4)	86 (21.2)	659 (27.3)	0.017
	VL <1.5 $\log_{10}$ copies/ml below set point	1195 (42.4)	194 (47.8)	1001 (41.5)	
	VL >1.5 $\log_{10}$ copies/ml below set point	880 (31.2)	126 (31.0)	754 (31.2)	
CD4 at the start of VF	Median(IQR), cells/mm^3	257 (145, 393)	260 (149, 407)	254 (142, 387)	0.092
RNA at the start of VF	Median(IQR), copies/mL	8200 (1829, 52672)	7200 (1801, 39000)	8928 (1841, 60205)	<.001

FU time contributed	Median(IQR), months	5 (2, 13)	8 (3, 17)	4 (1, 10)	<.001
Number of CD4	Median(IQR), months	3 (2, 5)	4 (2, 6)	3 (2, 5)	<.001
Time between CD4	Median(IQR), months	3 (1, 5)	4 (2, 5)	3 (1, 4)	<.001
Genotypic Sensitivity Score (GSS) during each episode[1] (Median, IQR)		1 (0, 3)	1 (0, 2)	2 (1, 3)	<.001

1. Calculated using the ANRS (v 23) interpretation system.

2. The set point could be estimated for 2820 episodes

Table 5.4. Characteristics of the study population according to the detection of resistance, per episode

		Total Episodes	No Resistance	Any resistance	P-value
		N (%)	N (%)	N (%)	
At baseline, per individual		5357	625	4732	
Gender	Male	4088 (76.3)	482 (77.1)	3606 (76.2)	0.613
	Female	1269 (23.7)	143 (22.9)	1126 (23.8)	
Ethnicity	White	3592 (68.2)	443 (72.3)	3149 (67.7)	0.022
	Non-white	1673 (31.8)	170 (27.7)	1503 (32.3)	
Risk Group	MSM	2862 (55.0)	382 (62.1)	2480 (54.0)	0.002
	PWID	485 (9.3)	50 (8.1)	435 (9.5)	
	Heterosexual	1726 (33.1)	167 (27.2)	1559 (34.0)	
	Other	134 (2.6)	16 (2.6)	118 (2.6)	
Subtype	B	3659 (72.2)	450 (75.8)	3209 (71.7)	0.039
	Non-B	1409 (27.8)	144 (24.2)	1265 (28.3)	
Hepatitis C	Yes	698 (9.1)	83 (9.7)	3891 (57.2)	<.001
	No	4461 (58.2)	569 (66.4)	615 (9.0)	
	Unknown	2502 (32.7)	205 (23.9)	2297 (33.8)	
Age (Median, IQR)	Years	38.5 (34.6, 42.9)	40.3 (35.9, 43.8)	38.3 (34.5, 42.8)	0.046
Baseline date (Median, IQR)	Year	12/00 (06/98-11/04)	12/03 (11/99-08/07)	09/00(05/98-06/04)	<.001
During FU, per episode		7661	857	6804	
Viral suppression[2]	VL above set point	745 (26.4)	160 (35.2)	585 (24.7)	<.001
	VL <1.5 $\log_{10}$ copies/ml below set point	1195 (42.3)	183 (40.3)	1012 (42.8)	
	VL >1.5 $\log_{10}$ copies/ml below set point	880 (31.2)	111 (24.5)	769 (32.5)	
CD4 at the start of VF	Median (IQR), cells/mm^3	257 (145, 393)	273 (158, 406)	255 (143, 392)	0.046

RNA at the start of VF	Median (IQR), copies/mL	8200 (1829, 52672)	22600 (3155, 102265)	7400 (1755, 45266)	<.001
FU time contributed	Median (IQR), months	5 (2, 13)	3 (1, 8)	6 (2, 13)	<.001
Number of CD4 measurements	Median (IQR), months	3 (2, 5)	2 (2, 4)	3 (2, 5)	<.001
Time between CD4 measurements	Median (IQR), months	3 (1, 5)	2 (1, 4)	3 (2, 5)	<.001
Genotypic Sensitivity Score (GSS) during each episode[1]	(Median, IQR)	1 (0, 3)	3 (3, 4)	1 (0, 3)	<.001

1. Calculated using the ANRS (v 23) interpretation system.

2. The set point could be estimated for 2820 episodes

A large number of different combinations of antiretroviral drugs were used for the duration of the VF episodes (Table 5.5). The vast majority of the episodes (84.5%) were taking place when individuals were receiving more than 3 drugs, although 15.6% of episodes occurred while individuals were receiving mono or dual therapy. Of those receiving exactly 3 drugs, the most common combination was one NNRTI and 2 NRTI. The most common combination was Zidovudine, Lamivudine and Nevirapine, used in 3% of the episodes. This was followed by dual therapy consisting of Lamivudine and Stavudine or Zidovudine and Lamivudine. The regimens used likely reflect the early calendar years included in the analysis.

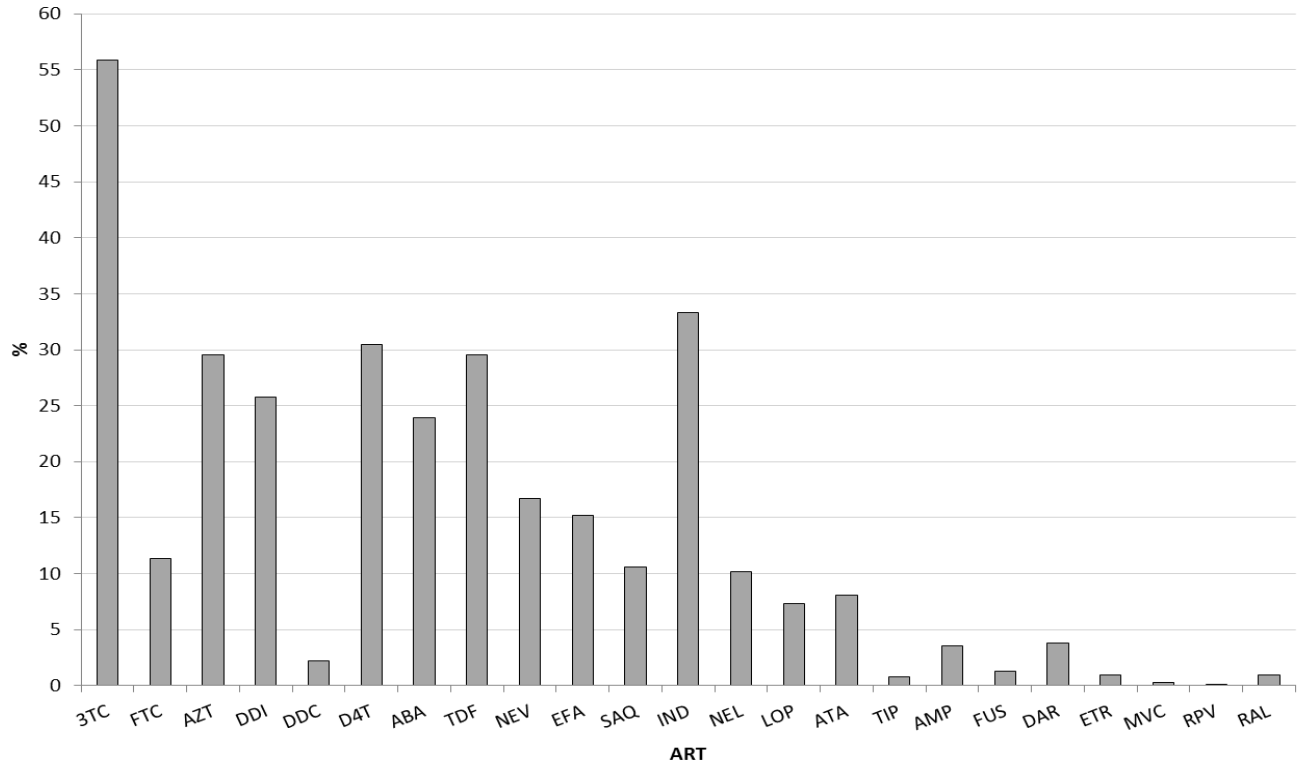
Table 5.5. Most commonly used regimens[1], per episode

	N	%
Zidovudine+Lamivudine+Nevirapine	232	3.03
Lamivudine + Stavudine	228	2.98
Zidovudine + Lamivudine	211	2.75
Lamivudine + Stavudine + Nelfinavir	192	2.51
Tenofovir + Efavirenz + Emtricitabine	183	2.39
Tenofovir + Indinavir/r + Atazanavir + Emtricitabine	147	1.92
Zidovudine + Lamivudine + Efavirenz	141	1.84
Zidovudine + Lamivudine + Abacavir	140	1.83
Lamivudine + Stavudine + Nevirapine	129	1.68
Lamivudine + Stavudine + Indinavir	128	1.67
Zidovudine + Lamivudine + Nelfinavir	125	1.63
Tenofovir + Indinavir/r + Emtricitabine	115	1.5
Didanosine + Stavudine + Nevirapine	111	1.45
Zidovudine + Lamivudine + Indinavir	111	1.45
Zidovudine + Lamivudine + Indinavir/r	98	1.28
Didanosine + Stavudine + Efavirenz	87	1.14
Lamivudine + Abacavir +Nevirapine	87	1.14
Zidovudine + Didanosine	86	1.12
Didanosine + Stavudine	85	1.11
Tenofovir + Indinavir/r + Darunavir/r + Emtricitabine	80	1.04
Lamivudine + Abacavir + Efavirenz	78	1.02

1. *Only regimens used during more than 1% of episodes are reported*

The prevalence of individual drugs used can be seen in Figure 5.5. Lamivudine (3TC) was used most commonly, in just over 55% of the episodes. This was followed by Indinavir, Stavudine and Atazanavir.

Figure 5.5. Individual drugs used during each episode[1]



1. 3TC=Lamivudine, FTC=Emtricitabine, AZT=Zidovudine, DDI=Didanosine, D4T=Stavudine, ABA=Abacavir, TDF=Tenofovir, NEV=Nevarapine, EFA=Efavirenz, SAQ=Saquinavir, IND=Indinavir, NEL=Nelfinavir, LOP=Lopinavir, ATA=Atazanavir, TIP=Tipranavir, AMP=Fos-amprenavir, FUS=T-20 (Fuseon), DAR=Darunavir, ETR=Etravirine, MVC=Maraviroc, RPV=Rilpivirine, RAL=Raltegravir

5.4.2. Resistance prevalence

At least one resistance mutation was detected in 6,804/7,661 episodes (88.8%). Of those episodes during which no resistance was detected, 9.8% had only minor PI mutations and 1.3% had no mutations detected whatsoever. The prevalence of any NRTI resistance was 68.0%, any NNRTI resistance 58.2% and any PI resistance (excluding minor PI mutations) 51.0%. Estimates of class-specific resistance prevalence according to the different interpretation systems are also provided in Figure 5.6. The prevalence appeared reasonably similar across the different identification systems, although the Rega system appeared to provide a lower estimate of the prevalence of PI mutations as compared to the other systems.

Four mutations in the RT (G190T, T69S, T66i, W71i) and four in the PI (V32i, I47I, G48Q, G73F) were not detected in the dataset, and 82 RT mutations and 62 PI mutations were detected at a frequency of less than 1%. These were not evaluated as individual mutations in this analysis. The prevalence of the remaining 55 RT mutations and 64 PI mutations can be seen in Figure 5.7 and Figure 5.8. In the RT gene, the M184V substitution that confers resistance to NRTIs, typically 3TC and/or FTC, was most commonly detected, followed by the K103N mutation that confers resistance to most NNRTIs. In the PR gene, minor PR mutations were the most common, with the L63P substitution detected in just over 55% of episodes. The most common major PI mutation was L90M (10%).

Figure 5.6. Prevalence of any and class-specific resistance according to the IAS (2015), Stanford (2015), Rega (2013) and ANRS (2015) interpretation systems, in 7661 viral failure episodes

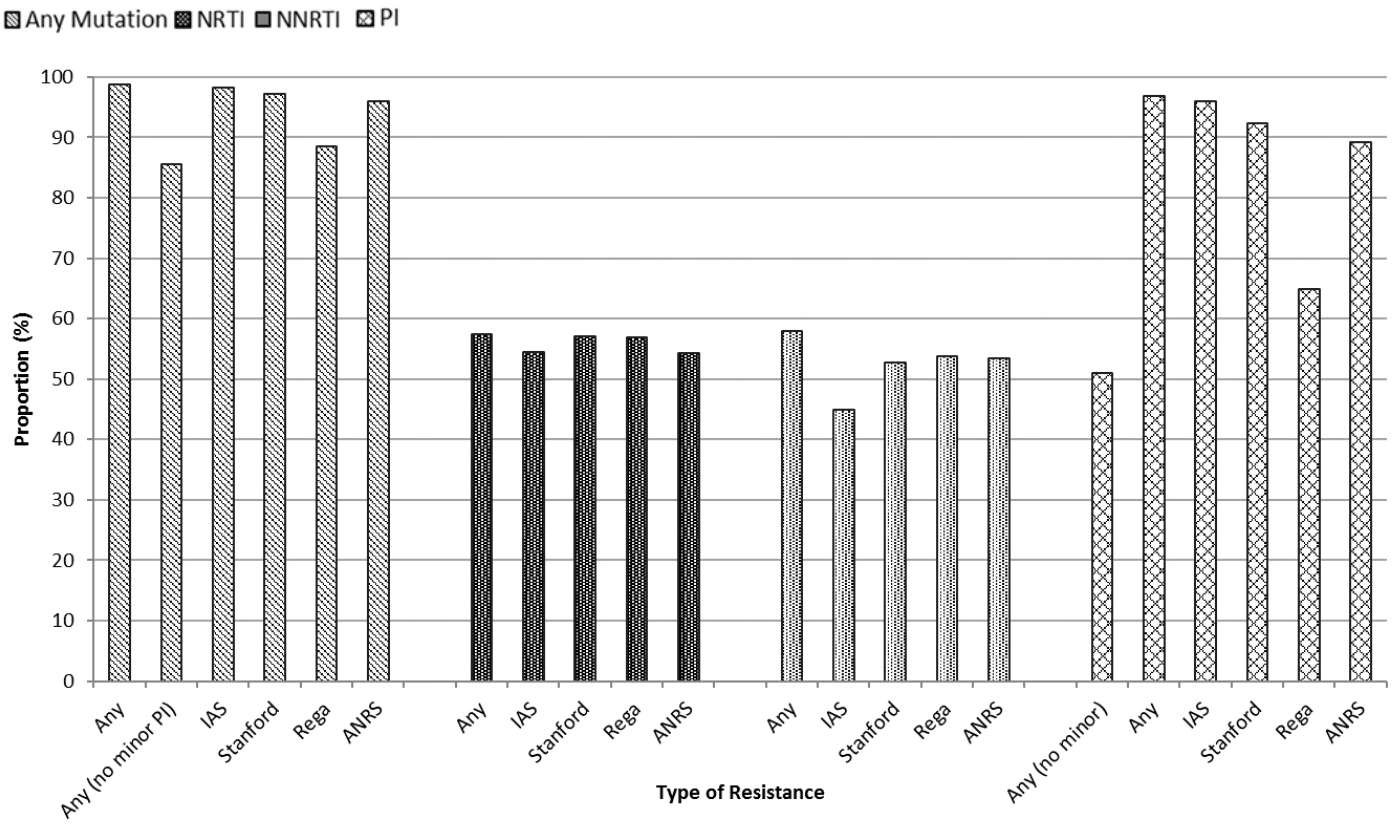


Figure 5.7. Prevalence of mutations in the RT gene detected in >1% of episodes, in 7661 VF episodes

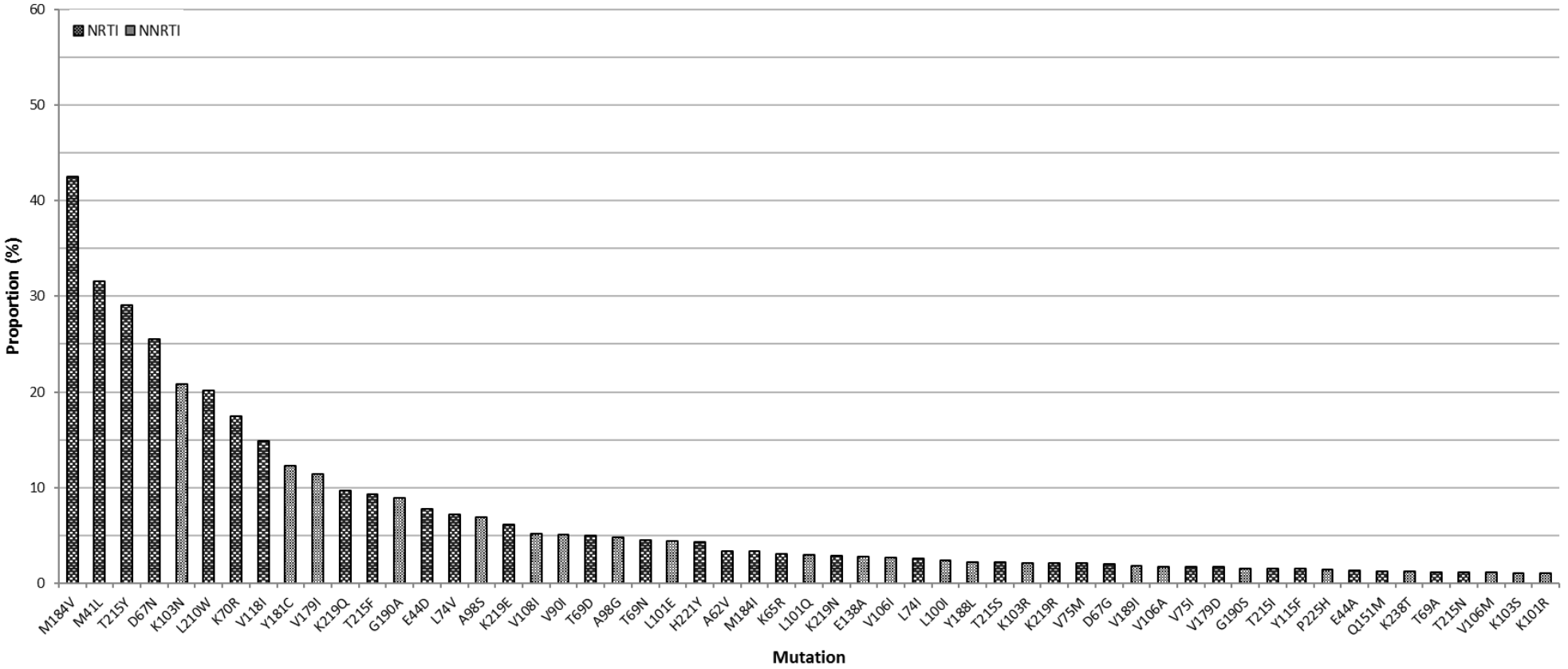
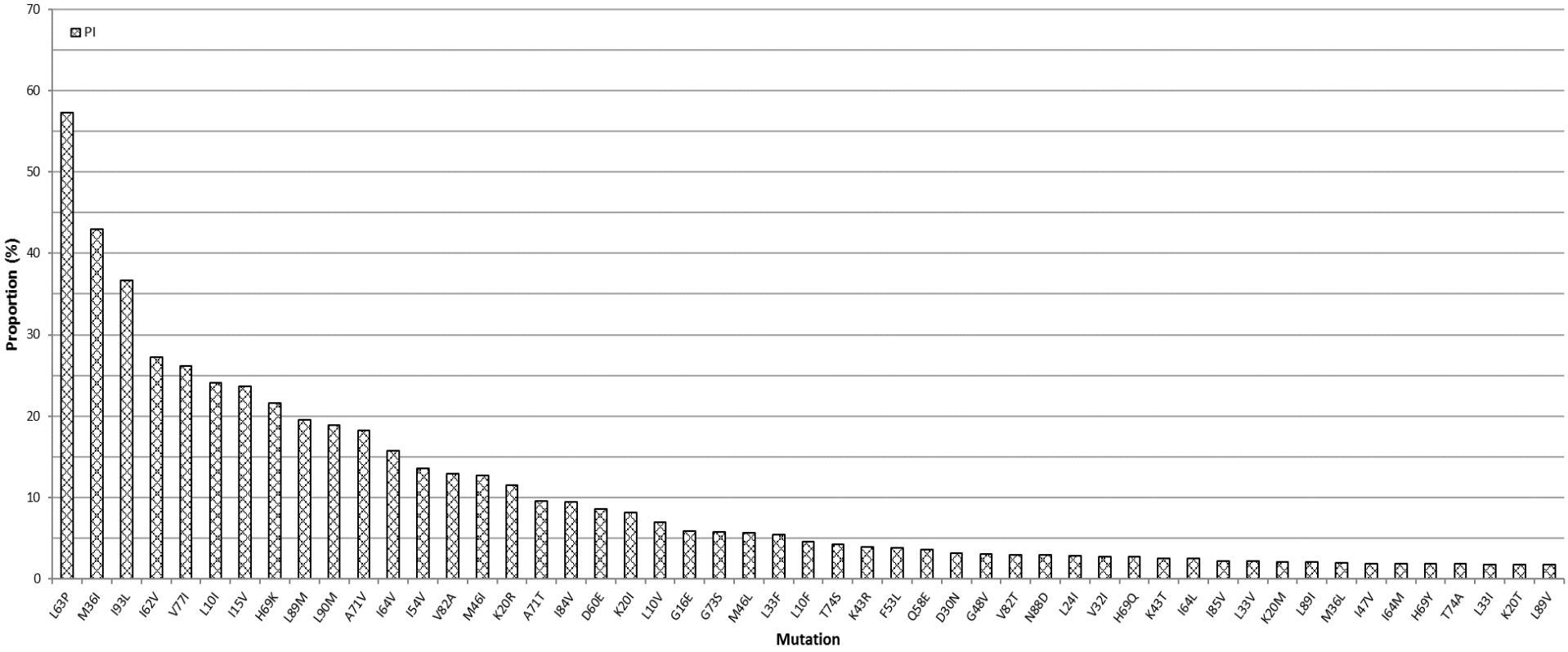


Figure 5.8. Prevalence of mutations in the PI gene detected in >1% of episodes, in 7661 VF episodes

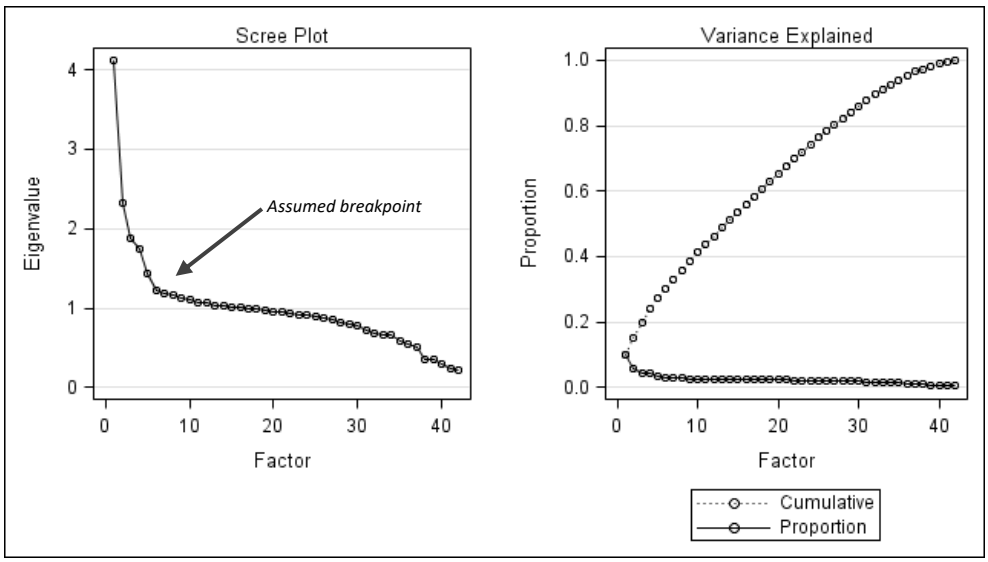


5.4.3. Clusters of mutations

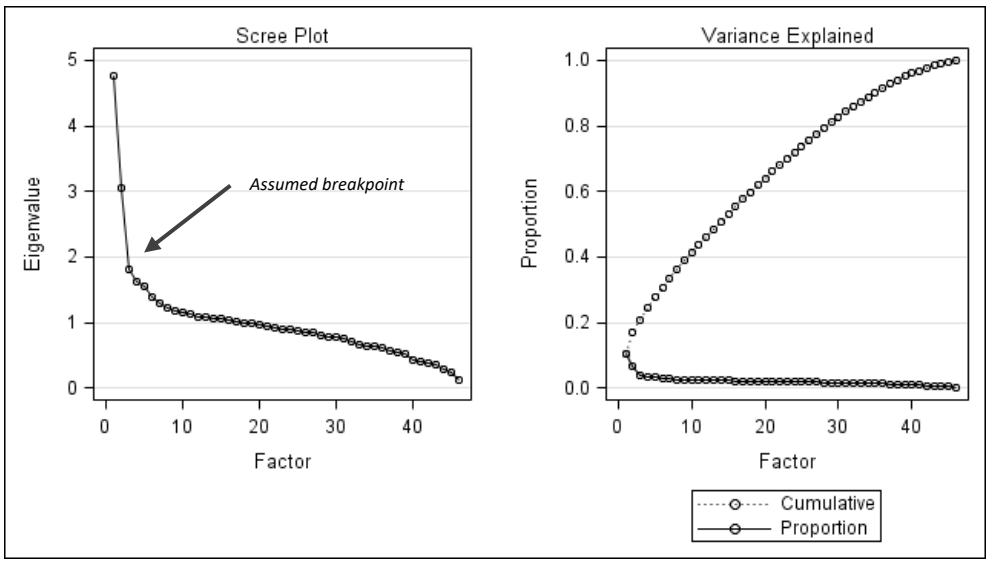
The scree plots I used to decide how many principal components (PC's) to retain for the RT and PI PCA can be seen in Figure 5.9. These plots showed approximate breakpoints after five (RT) and two (PI) principal components (PC's) (Figure 5.9), and therefore I decided to retain five RT components and two PI components. In total, these factors explained a low proportion of the total variance: 27.7% and 17.3% respectively.

Figure 5.9. Scree plot for RT (a) and PI (b) mutations

(a)



(b)



The extracted factors were subjected to a varimax rotation (Chapter 2, page 100) to aid the interpretability of the results, and the rotated factor patterns are shown in Table 5.6-Table 5.7 (PI mutations). Briefly, these tables show how strongly each mutation correlates with the extracted PC. The strength of the correlation, the loadings, have been rounded and multiplied by 100 in order to ease the interpretation of the tables. Loadings with values between 30 and 40 are highlighted in light gray, between 40 and 70 in medium gray and above 70 in dark gray. For simplicity, extracted components are referred to as clusters from now on. The loadings are used to describe the clusters characteristics. However, for the extraction of individual scores all mutations contribute as described in Chapter 2, page 100.

The 1st RT cluster primarily consisted of substitutions in positon 215, 67, 41, 210, 219, 44, 118 and 70. These substitutions correspond roughly to the TAM mutations, a well-characterised cluster of mutations. The strongest contributions came from TAM-1 mutations (210, 215 and 41) as well as from substitutions in position 67. The 2nd RT cluster corresponded to the 151M cluster, with substitutions in position 151, 116, 77, 75. There were also some weak contributions from substitutions in position 62 and 65. The 3rd RT cluster corresponded weakly to the TAM2 cluster (substitutions in positon 70, 219 and, weakly, 67). The mutations that formed part of the 4th and 5th RT clusters did not represent any already known mutation patterns. Substitutions in positions 74, 221 and 181 contributed to the 4th RT cluster, with weak contributions from substitutions in position 103, 108 and 100. Substitutions in position 101, 103 and 225 contributed to the 5th. The 4th RT cluster therefore represents broad NNRTI resistance (221 and 181), but also resistance to abacavir and didanosine. The 5th cluster represents general resistance to NNRTI drugs.

Major PI mutations in positions 46, 48 53, 54, 58, 82, 84 and 90 all contributed to the 1st PI cluster, as did minor mutations in position 10, 24, 33, 53, 64, 71 and 73. Together, these substitutions confer broad PI resistance. The 2nd PI cluster consisted of 4 minor PI mutations in position 20, 36, 69 and 89, with some weak contributions from substitutions in position 16 and 15.

Table 5.6. Rotated Factor Pattern for RT mutations

	Component 1 *TAM*	Component 2 *151 Complex*	Component 3 *TAM2*	Component 4 *Cross NNRTI + ABC/DDI*	Component 5 *Cross NNRTI*
r40F	17	-4	-8	-1	0
r41L	72	-22	-35	1	2
r44AD	54	-14	-39	5	-9
r62V	11	37	-14	5	-1
r65ENR	-3	37	4	23	-17
r67EGHNS	72	-11	31	-31	2
r68ins	4	3	-3	-1	-1
r69ADGNS	35	-3	25	-18	-6
r69ins	4	2	2	1	-3
r70EGNQRST	42	2	65	-39	8
r74IV	31	6	7	48	26
r75AILMST	31	40	-13	-2	3
r77I	12	66	-22	-22	10
r90I	2	8	11	6	2
r98GS	12	-1	2	6	-9
r100IV	12	1	6	31	51
r101EHINPQRT	24	17	18	25	-38
r103HNRST	17	10	17	39	48
r106AIM	6	9	7	8	-22
r108I	22	15	7	32	8
r115F	5	43	1	13	-8
r116Y	9	76	-17	-30	11

r118I	56	-12	-33	-4	-7
r138AGKQRS	-2	6	7	7	-10
r151LM	10	77	-14	-28	10
r179DEFILMT	9	5	18	9	-16
r181CFGISV	27	23	19	42	-31
r184IV	37	2	3	7	17
r188CFHL	8	-2	-2	6	-3
r189I	6	3	-5	14	2
r190ACEQSTV	30	21	20	25	-40
r191I	3	-3	-5	-1	-8
r210W	67	-20	-48	6	-5
r215ACDEFGHILNSVY	77	-21	-12	-8	4
r219EHNQRW	59	0	54	-28	7
r221Y	16	15	14	42	-15
r225H	-3	5	8	21	43
r227CL	1	-1	5	-4	-4
r230ILV	2	2	8	7	10
r234I	0	-1	-2	-1	0
r238NT	12	0	8	8	13
r318F	3	-1	8	2	10
r348I	1	-1	-3	1	2

Table 5.7. Rotated Factor Pattern for PI mutations

	Component 1 ***Cross PI***	Component 2 ***Accessory/Non-B***
p10CFIMRV	63	3
p11IL	14	7
p15AV	-9	37
p16E	-4	32
p20IMRTV	28	53
p23I	10	0
p24FIM	35	2
p30N	-1	-13
p32IL	25	-1
p33FIMV	31	1
p34QV	22	-2
p35GN	7	15
p36ILV	9	75
p38W	7	0
p41IT	2	6
p43TR	13	-3
p45I	2	-2
p46ILV	65	-6
p47AIV	24	1
p48ALMQSTV	36	0
p50LV	14	5
p53LWY	41	1
p54ALMSTV	78	9
p58E	30	5
p60E	6	7
p62V	27	-16
p63IP	26	-44
p64LMV	63	12
p66F	13	3
p69IKNQRY	-11	76
p70E	3	1
p71ILTV	57	-27
p73ACFSTV	40	-16
p74AEPS	17	23
p76V	20	4
p77I	6	-47
p82ACFGLMST	71	3
p83D	9	10
p84ACV	51	-4
p85V	20	-3
p88DGST	5	-10
p89IMRTV	-9	83
p90M	60	-14
p93LM	10	-8

5.4.4. CD4 count changes over time

In univariable analysis, the estimated average CD4 decline was 20 cells/mm^3/year (95%CI=-22; -17) in the entire population. The overall CD4 decline according to absolute levels of current VL as well as levels of viral suppression can be seen in Table 5.8. CD4 declines were somewhat steeper at very high viral loads (>100,000 copies/mL; p=0.01), but at lower viral loads no large differences according to strata of current VL were evident. CD4 declines were less steep (although still not negligible at 23 cells/mm^3 loss per year) when current VL levels were below pre-ART set-point values (p=0.008, Table 5.8). These results are in slight contrast with some previous findings (364,615), and possible reasons for these discrepancies are outlined in the discussion .

Table 5.8. CD4 count (cells/mm^3) decline per year in overall study population and according to time-updated VL levels and time-updated VL levels compared to viral set point

	Unadjusted		**Adjusted[1]**	
	Slope (95% CI)	P interaction[2]	Slope (95% CI)	P interaction[2]
Absolute VL		0.010		0.012
<1000	-11.25 (-16.33; -6.16)		-11.47 (-16.83; -6.11)	
1000-5000	-10.95 (-14.13; -7.77)		-11.22 (-14.61; -7.82)	
5000-10000	-9.80 (-13.25; -6.35)		-10.48 (-14.13; -6.83)	
10000-20000	-9.86 (-13.38; -6.34)		-10.82 (-14.55; -7.09)	
20000-100000	-12.64 (-15.83; -9.44)		-13.31 (-16.70; -9.92)	
>100000	-17.21 (-21.07; -13.35)		-18.00 (-22.03; -13.98)	
VL in relation to set-point (N=2820)				
		0.066		0.008
Not supressed	-29.69 (-36.57; -22.81)		-33.67 (-40.92; -26.42)	
Somewhat supressed	-22.75 (-28.66; -16.84)		-23.78 (-30.00; -17.56)	
Supressed	-25.14 (-32.67; -17.61)		-26.07 (-33.91; -18.24)	

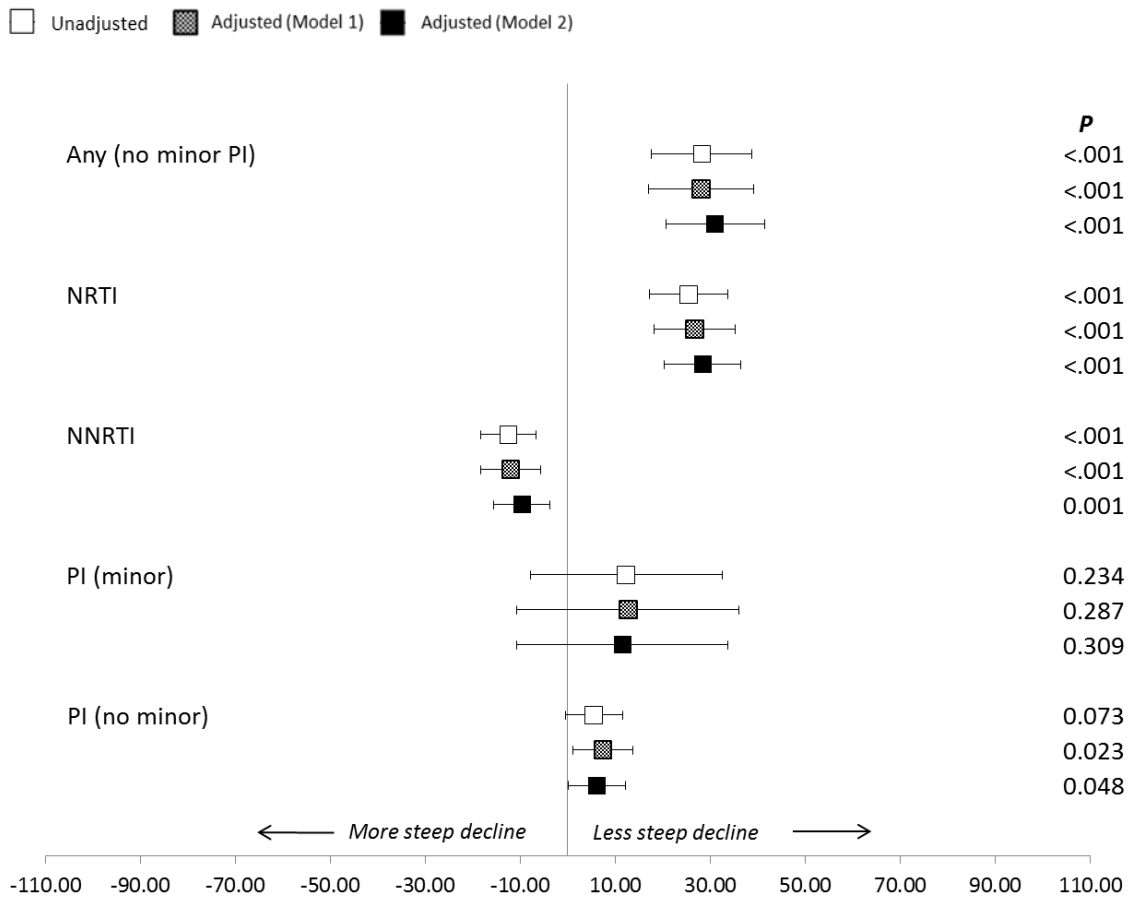
1. Adjusted for drug type, age, hepatitis C, risk-group, subtype and GSS. 439 individuals were excluded due to missing values for subtype and risk group.

2. Testing the null hypothesis that the slope is the same in each RNA strata.

5.4.5. Influence of any and class-wide resistance on CD4 slopes

Figure 5.10 shows the estimated difference in annual CD4 decline according to the detection of any resistance of resistance associated with specific drug classes. CD4 counts declined less rapidly during episodes where drug resistance was detected compared to episodes where resistance was not detected, with an estimated difference of 28 (18-39) cells/mm^3/year after comparing decline in people with resistance (-17, 95%CI=-20; -15 cells/mm^3/year) to that among those with no resistance (-46, 95%CI=-56; -36) cells/mm^3/year, interaction p<0.001). These findings persisted after adjustment for the pre-specified confounders (difference=28 [17-39] cells/mm^3/year, p<0.001) and after further adjustments for VL (difference=32 [21-42] cells/mm^3/year, p<0.001, Figure 5.10).

Figure 5.10. Difference in annual CD4 count changes (cells/mm^3) according to the detection of any and class of drug resistance present[1,2,3,4]



1. The first category, "Any (no minor PI) compares those without resistance to those with at least 1 DRM from any classification system, excluding IAS minor PI mutations

2. The consequent four categories compare those with at least 1 DRM from the specified drug class to those with any other DRM. Individuals without resistance were excluded from these comparisons

3. Model 1 adjusted for age, hepatitis C, risk group and subtype. Model 2 additionally adjusted for viral load (log-scale, continuous).

4. Separate models were constructed for each exposure

Among individuals with detected drug resistance, CD4 counts declined less rapidly during episodes where NRTI resistance was detected compared to episodes where NRTI resistance was not detected (-15 (-18; -12) v -40 (-48; -33) cells/mm^3/year, interaction p<0.001). Adjustment for pre-specified confounders and VL did not change these estimates markedly (Figure 5.10). There was also evidence that CD4 counts declined faster during episodes where NNRTI resistance was detected in both univariable (-22 (-26; -19) v -10 (-15; -5) cells/mm^3/year, interaction p<0.001) analyses and after adjustment (Figure 5.10).

There was no evidence to suggest that CD4 decline differed according to the detection of PI resistance including minor PI mutations (Figure 5.10). However, there was some weak evidence suggesting that CD4 decline was less steep during episodes where major PI mutations were detected, both in univariable (difference=6 (-1-12) cells/mm^3/year, p=0.07) and multivariable models (difference=8 (1-14) cells/mm^3/year, p=0.02 in model 1, difference= 6 (-0; 12) cells/mm^3/year, p=0.05 in model 2).

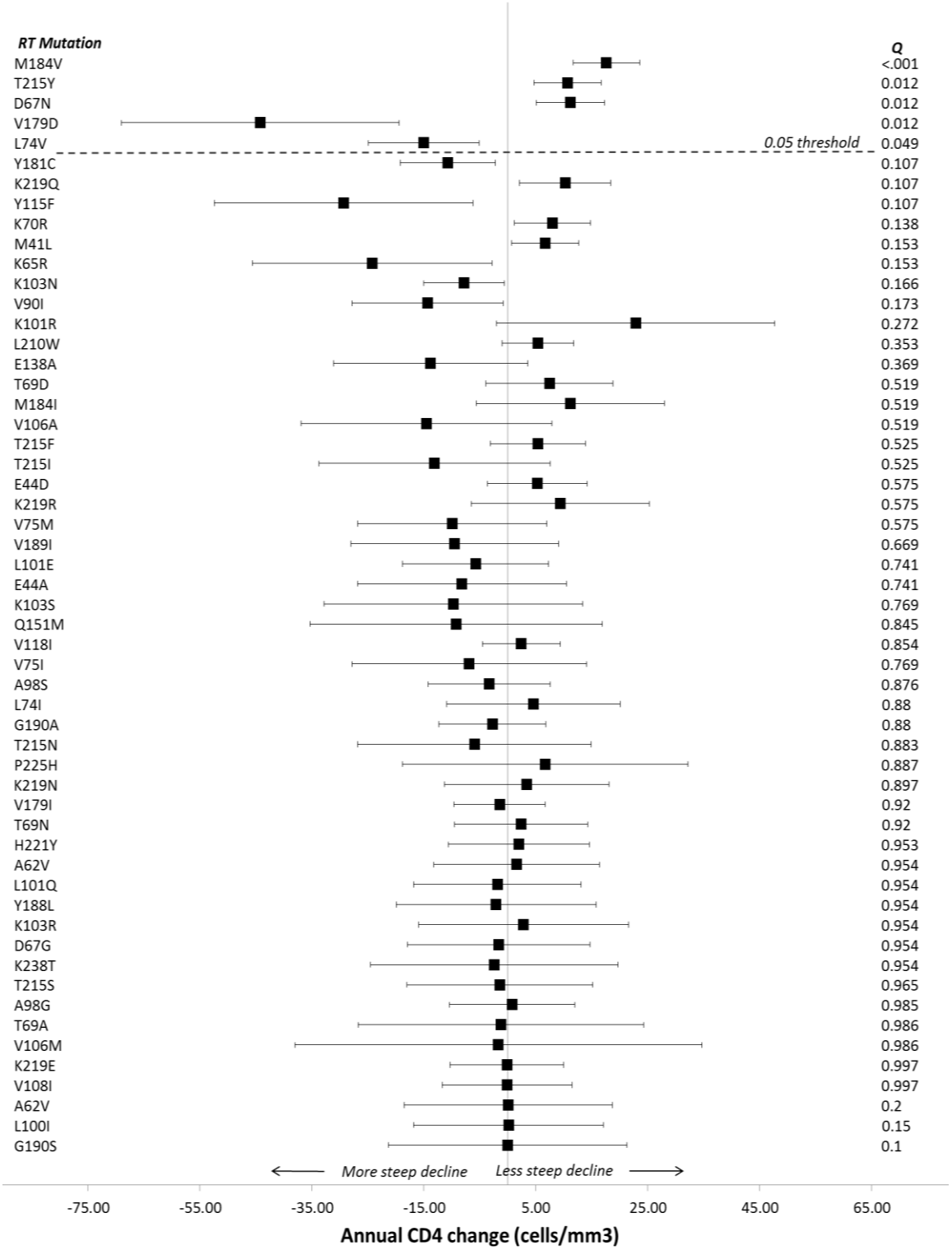
5.4.6. Influence of individual mutations on CD4 slopes

The difference in CD4 decline between episodes with and without a given mutation after adjustment for pre-specified confounders is shown in Figure 5.11. Mutations are ranked with increasing q-values from top (most significant associations after controlling for multiple testing) to the bottom (least significant associations after controlling for multiple testing). After correcting for multiple testing, the strongest association was found for M184V, with episodes where M184V was detected experiencing somewhat less steep CD4 declines (difference= 17 (11-23) cells/mm^3/year, q<0.001). These findings did not change markedly after additional adjustment for VL (difference=16 (10-21) cells/mm^3/year, q<0.001, Figure 5.12). There was also reasonable evidence that the T215Y and D67N mutations were associated with less steep CD4 declines both before (difference=11 (5-17) and 11 (5-17) respectively, both q=0.012) and after (difference=10 (4-16) and 12 (6-17), q=0.019 and 0.003 respectively) adjustment for VL. One NNRTI mutation, V179D, was associated with steeper CD4 declines, although there was uncertainty around the exact magnitude of the effect (difference=-44 (-69; -19) cells/mm^3/year, q=0.012, Figure 5.12). There was also some weak evidence (q=0.05) suggesting that those with the L74V mutation experienced somewhat steeper CD4 declines, but the association was no longer significant after controlling for VL (Figure 5.12).

Of the PI mutations studied, two remained associated with a less steep CD4 decline after correction for multiple testing: V82A and I54V (Figure 5.13). The strongest association was

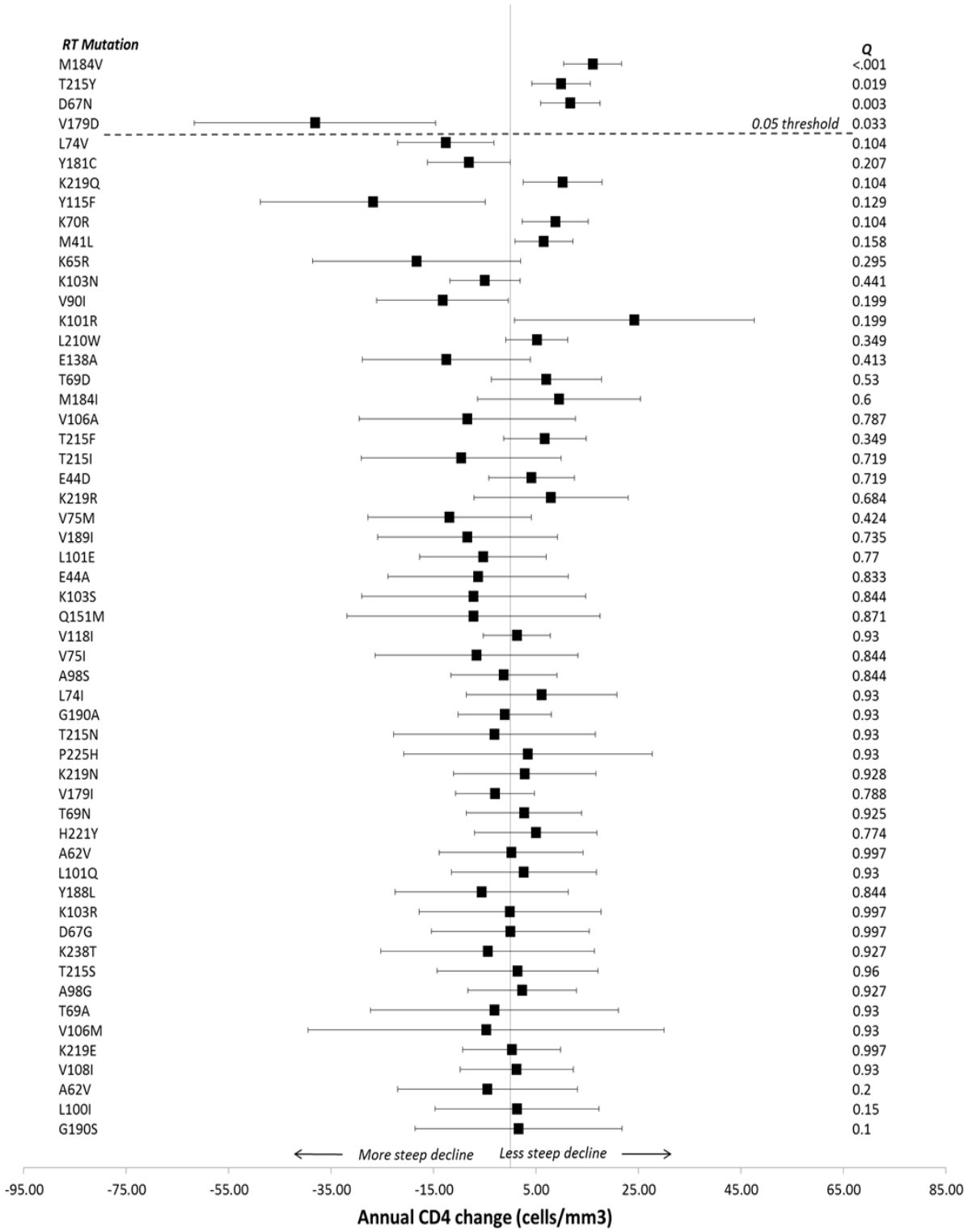
found for the V82A mutation, where CD4 decline was estimated to be 18 (10-25) cells/mm^3/year less steep during episodes where the mutation was detected compared to episodes without the mutation ($q<0.001$). CD4 decline was also 13 (5-20) cells/mm^3/year less steep during episodes where I54V was detected ($q=0.015$). Again, results were similar after further adjusting for VL in the model (Figure 5.14).

Figure 5.11. Difference in annual CD4 count changes according to the detection of RT mutations[1]



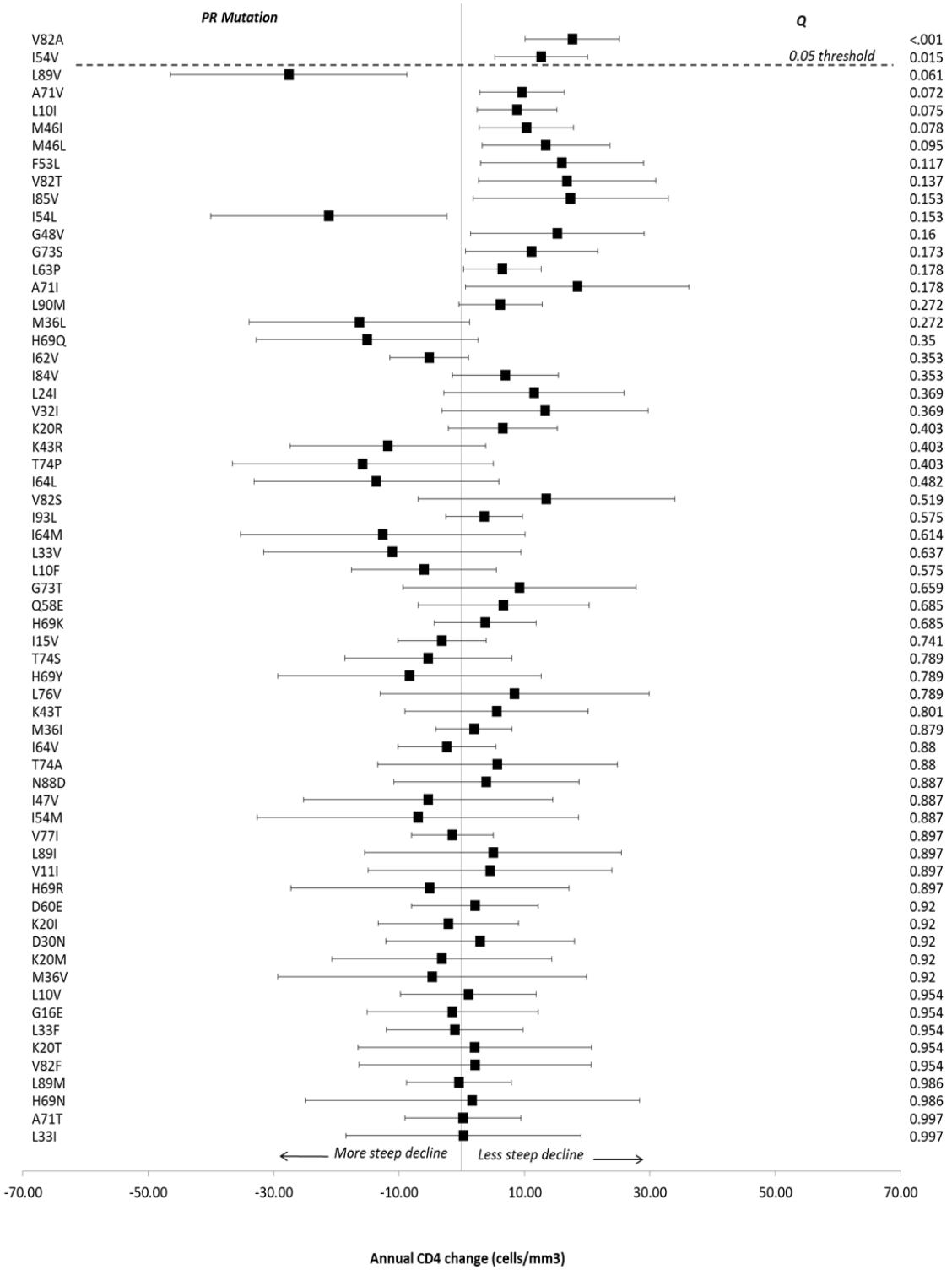
1. *Adjusted for age, hepatitis C, risk-group and subtype. 439 individuals were excluded due to missing values for subtype and risk group.*

Figure 5.12. Difference in annual CD4 count changes according to the detection of RT mutations (Model 2, adjusted for VL)[1,2]



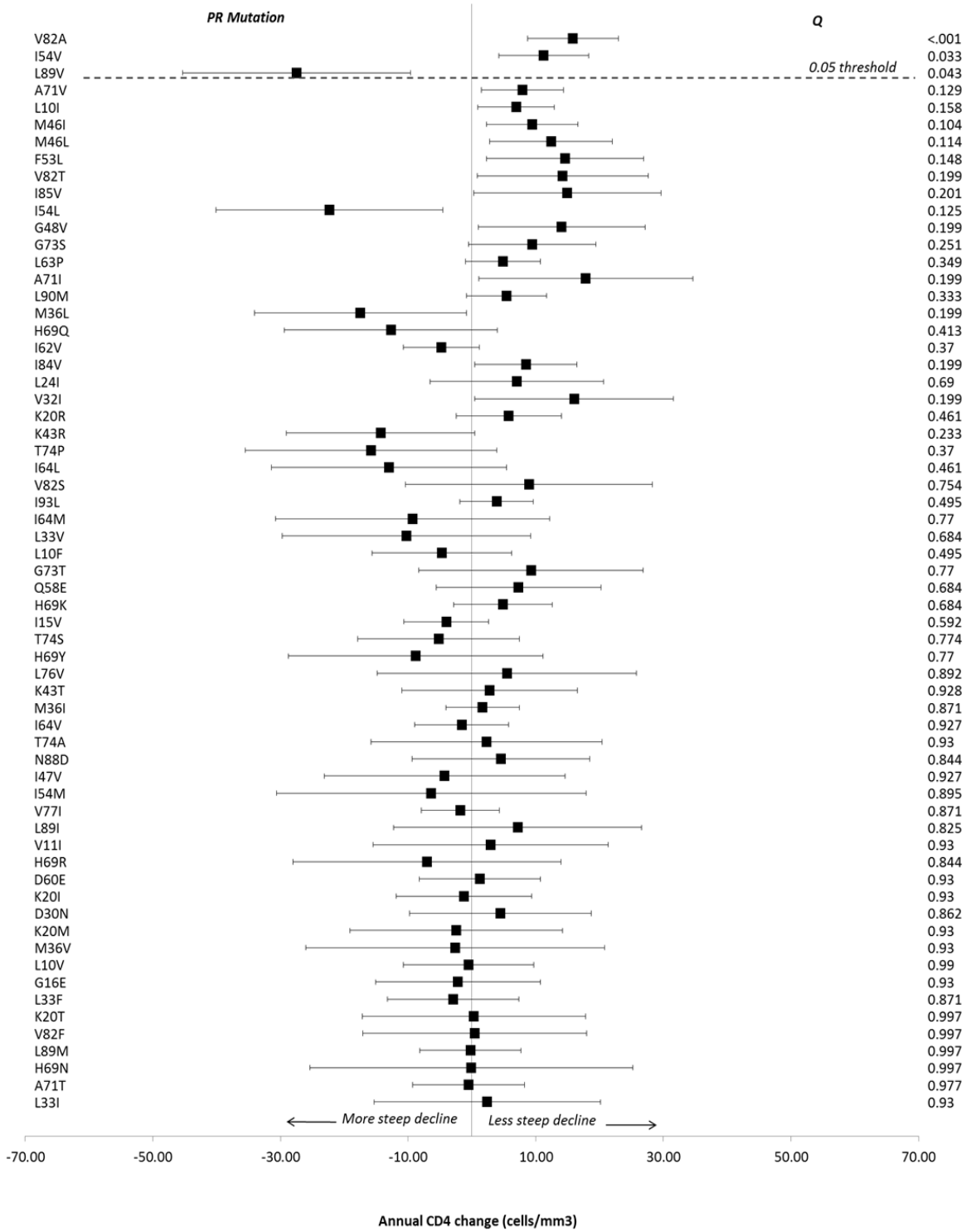
1. *Adjusted for age, hepatitis C, risk-group, subtype and VL . 439 individuals were excluded due to missing values for subtype and risk group.*
2. *For M184V, it was necessary to adjust for VL as a categorical variable due to convergence issues.*

Figure 5.13. Difference in annual CD4 count changes according to the detection of PI mutations[1]



1. *Adjusted for age, hepatitis C, risk-group and subtype . 439 individuals were excluded due to missing values for subtype and risk group.*

Figure 5.14. Difference in annual CD4 count changes according to the detection of PR mutations (Model 2, adjusted for VL)

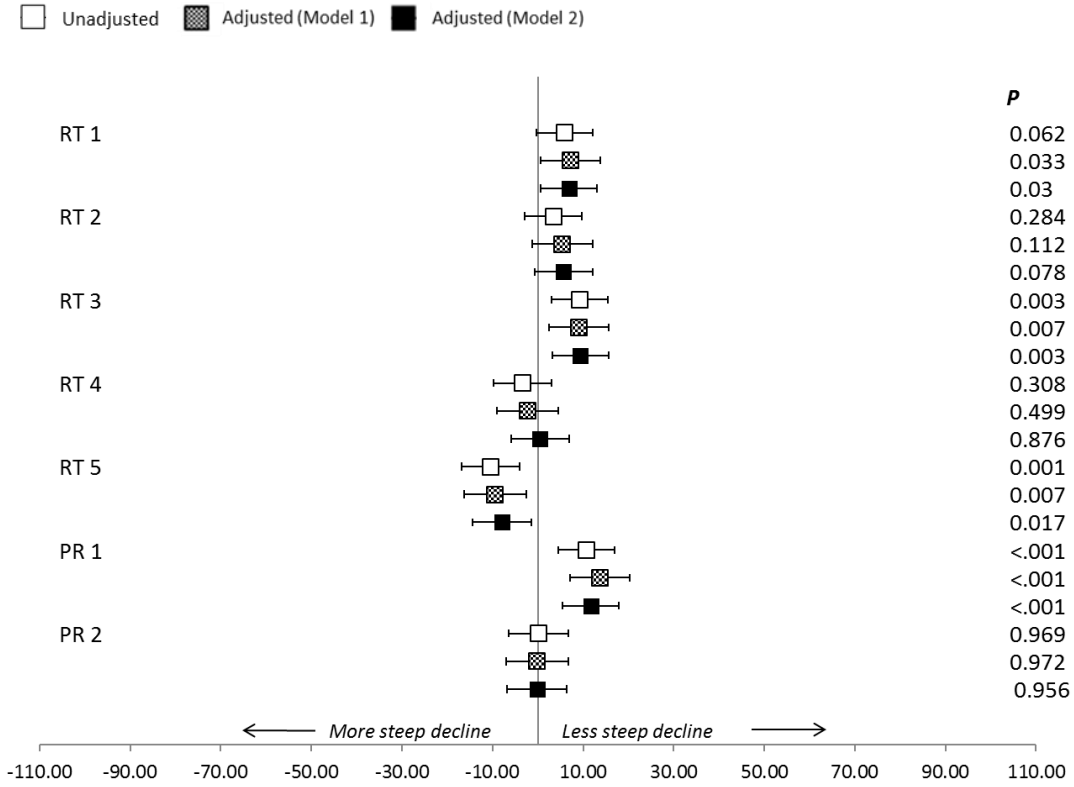


1. *Adjusted for age, hepatitis C, risk-group, subtype and VL . 439 individuals were excluded due to missing values for subtype and risk group.*
2. *For K20T and I93L, it was necessary to adjust for VL as a categorical variable due to convergence issues.*

5.4.7. The effect of mutation clusters on CD4 count change

The effect of the mutation clusters on CD4 count changes are shown in Figure 5.15. Individuals with scores in the highest quartile of the 3rd RT cluster (weakly corresponding to TAM-2 mutations) experienced reduced CD4 declines (difference=9 (2-16) cells/mm^3/year, p=0.007), as did individuals with scores in the highest quartile of the 1st PI cluster, which represented broad PI resistance (difference=14 (7-20) cells/mm^3/year, p<0.001). Individuals with scores in the highest quartile of the 5th RT cluster, which included K103N, experienced steeper CD4 declines (difference=-9 (-16; -3) cells/mm^3/year, p=0.007) compared to individuals with lower scores on this component. There was also some very weak evidence that scores in the highest quartile of the 1st RT cluster, corresponding roughly to TAM-1 mutations, were associated with somewhat reduced CD4 declines (p=0.03)

Figure 5.15. CD4 decline according to RT[1] and PC[2] clusters identified by the PCA[3]



1. *RT1= TAM1, RT2=151M, RT3=TAM2, RT4=NNRTI/NRT, RT5=NNRTI*
2. *PR1=Cross PI, PR2=Minor PI*
3. *Model 1 adjusted for age, hepatitis C, risk group and subtype. Model 2 additionally adjusted for viral load (log-scale, continuous). 439 individuals were excluded due to missing values for subtype and risk group.*

5.4.8. Sensitivity analyses

The results from the sensitivity analyses are shown in Table 5.9-Table 5.12 for any resistance, class-wide resistance, the significant DRM identified above and the PC components respectively. When restricting to episodes without very large VL variations (N=7,575 episodes, 98.8% of the original dataset) the point estimates and confidence intervals of main findings only shifted by a few cells/mm^3/year, and the conclusions remained unchanged. When excluding episodes with fewer than 3 CD4 counts (N=4613 episodes, 60.2% of the original dataset), the statistical significance of some of the findings was reduced. Specifically, the q-values associated with all individual mutations apart from M184V and V82A no longer met the 0.05 threshold. No other findings were materially different (Table 5.11). Adjusting for the class of drug (NNRTI, PI, NNRTI+PI, Other) received during the regimen did not affect any of the findings (Table 5.12).

Table 5.9. Summary of the main results in the original analysis[1]

	Unadjusted		Adjusted (1)		Adjusted (2)	
	Difference (95%CI)	P	Difference (95%CI)	P	Difference (95%CI)	P
N=7,661						
Any	26.39 (13.65 - 39.13)	<.001	28.11 (17.11 - 39.12)	<.001	31.07 (20.63 - 41.51)	<.001
Class-wide	Difference (95%CI)	P	Difference (95%CI)	P	Difference (95%CI)	P
NRTI	25.45 (17.19 - 33.71)	<.001	26.66 (18.12 - 35.20)	<.001	28.40 (20.28 - 36.52)	<.001
NNRTI	-12.47 (-18.35 - -6.60)	<.001	-11.99 (-18.23 - -5.75)	<.001	-9.59 (-15.50 - -3.67)	0.001
PI (no minor)	5.51 (-0.52 - 11.53)	0.073	7.40 (1.03 - 13.78)	0.023	6.09 (0.05 - 12.12)	0.048
PI (minor)	12.27 (-7.92 - 32.47)	0.234	12.71 (-10.69 - 36.11)	0.287	11.51 (-10.68 - 33.70)	0.309
Individual DRM	Difference (95%CI)	Q	Difference (95%CI)	Q	Difference (95%CI)	Q
M184V	14.46 (8.82 - 20.11)	<.001	17.61 (11.65 - 23.58)	<.001	16.03 (10.40 - 21.66)	<.001
T215Y	9.39 (3.74 - 15.04)	0.022	10.67 (4.67 - 16.66)	0.012	9.88 (4.20 - 15.56)	0.019
D67N	10.53 (4.80 - 16.27)	0.013	11.15 (5.03 - 17.27)	0.012	11.65 (5.86 - 17.45)	0.003
L179D	-41.04 (-64.31 - -17.77)	0.016	-44.23 (-69.06 - -19.41)	0.012	-38.15 (-61.69 - -14.60)	0.033
L74V	-15.85 (-25.19 - -6.50)	0.021	-15.00 (-24.88 - -5.12)	0.049	-12.62 (-21.98 - -3.26)	0.104
V82A	14.51 (7.45 - 21.58)	0.003	17.64 (10.13 - 25.14)	<.001	15.83 (8.72 - 22.94)	<.001
I54V	11.00 (4.06 - 17.94)	0.032	12.69 (5.29 - 20.09)	0.015	11.26 (4.24 - 18.28)	0.033
Clusters	Difference (95%CI)	P	Difference (95%CI)	P	Difference (95%CI)	P
RT 1	5.90 (-0.29 - 12.09)	0.062	7.17 (0.57 - 13.78)	0.033	6.90 (0.65 - 13.15)	0.03
RT 2	3.42 (-2.84 - 9.69)	0.284	5.43 (-1.27 - 12.14)	0.112	5.71 (-0.63 - 12.05)	0.078
RT 3	9.28 (3.11 - 15.45)	0.003	9.14 (2.53 - 15.75)	0.007	9.41 (3.16 - 15.67)	0.003
RT 4	-3.32 (-9.70 - 3.07)	0.308	-2.33 (-9.10 - 4.43)	0.499	0.51 (-5.90 - 6.92)	0.876

RT 5	-10.45 (-16.87 - -4.03)	0.001	-9.41 (-16.25 - -2.56)	0.007	-7.87 (-14.36 - -1.38)	0.017
PI 1	10.67 (4.47 - 16.87)	<.001	13.69 (7.06 - 20.31)	<.001	11.67 (5.39 - 17.95)	<.001
PI 2	0.13 (-6.40 - 6.66)	0.969	-0.13 (-7.02 - 6.77)	0.972	-0.18 (-6.73 - 6.36)	0.956

1. Adjusted for age, hepatitis C, risk-group, subtype (1) and VL (2). 439 individuals were excluded due to missing values for subtype and risk group.

Table 5.10. Sensitivity 1: Excluding large VL variation[1]

	Unadjusted		Adjusted (1)		Adjusted (2)	
	Difference (95%CI)	P	Difference (95%CI)	P	Difference (95%CI)	P
N=7,575						
Any	29.94 (19.15 - 40.74)	<.001	29.85 (18.62 - 41.07)	<.001	32.13 (21.45 - 42.82)	<.001
Class-wide	Difference (95%CI)	P	Difference (95%CI)	P	Difference (95%CI)	P
NRTI	25.26 (16.91 - 33.61)	<.001	-14.61 (-17.86 - -11.36)	<.001	-5.35 (-8.47 - -2.24)	<.001
NNRTI	-12.00 (-17.91 - -6.08)	<.001	-21.95 (-25.70 - -18.19)	<.001	-12.24 (-15.83 - -8.65)	0.002
PI (no minor)	5.37 (-0.70 - 11.44)	0.083	-15.71 (-19.40 - -12.03)	0.026	-7.12 (-10.64 - -3.60)	0.057
PI (minor)	13.30 (-7.14 - 33.73)	0.202	-17.78 (-20.87 - -14.70)	0.241	-8.80 (-11.76 - -5.85)	0.296
Individual DRM	Difference (95%CI)	Q	Difference (95%CI)	Q	Difference (95%CI)	Q
M184V	14.17 (8.50 - 19.84)	<.001	17.27 (11.26 - 23.28)	<.001	15.54 (9.85 - 21.23)	<.001
T215Y	9.53 (3.86 - 15.21)	0.024	10.73 (4.70 - 16.76)	0.011	9.89 (4.16 - 15.62)	0.021
D67N	10.61 (4.86 - 16.37)	0.012	11.13 (4.98 - 17.27)	0.011	11.59 (5.75 - 17.43)	0.004
L179D	-40.95 (-64.16 - -17.75)	0.016	-44.22 (-68.98 - -19.46)	0.011	-38.06 (-61.62 - -14.50)	0.036
L74V	-15.42 (-24.79 - -6.05)	0.025	-14.59 (-24.50 - -4.68)	0.064	-12.90 (-22.32 - -3.48)	0.096
V82A	14.77 (7.70 - 21.84)	0.003	17.92 (10.41 - 25.43)	<.001	16.08 (8.94 - 23.22)	<.001
I54V	10.78 (3.82 - 17.73)	0.04	12.43 (5.01 - 19.85)	0.02	10.90 (3.84 - 17.96)	0.045
Clusters	Difference (95%CI)	P	Difference (95%CI)	P	Difference (95%CI)	P
RT 1	5.56 (-0.65 - 11.77)	0.079	6.73 (0.10 - 13.36)	0.047	6.38 (0.08 - 12.67)	0.047
RT 2	3.21 (-3.10 - 9.52)	0.319	5.20 (-1.55 - 11.96)	0.131	5.16 (-1.26 - 11.58)	0.115
RT 3	9.73 (3.54 - 15.92)	0.002	9.52 (2.89 - 16.15)	0.005	9.80 (3.51 - 16.10)	0.002
RT 4	-3.47 (-9.88 - 2.93)	0.288	-2.59 (-9.38 - 4.21)	0.456	0.04 (-6.41 - 6.50)	0.989

RT 5	-9.90 (-16.33 - -3.46)	0.003	-8.86 (-15.73 - -1.99)	0.011	-7.44 (-13.97 - -0.90)	0.026
PI 1	10.25 (4.04 - 16.47)	0.001	13.21 (6.56 - 19.85)	<.001	11.23 (4.90 - 17.55)	<.001
PI 2	0.11 (-6.45 - 6.67)	0.973	-0.09 (-7.03 - 6.84)	0.979	-0.02 (-6.62 - 6.58)	0.996

1. Adjusted for age, hepatitis C, risk-group, subtype (1) and VL (2). 439 individuals were excluded due to missing values for subtype and risk group.

Table 5.11. Sensitivity 1: Only including viral failure episodes in which 3 or more CD4 counts measurements were available[11]

	Unadjusted		Adjusted (1)		Adjusted (2)	
	Difference (95%CI)	P	Difference (95%CI)	P	Difference (95%CI)	P
N=4,613						
Any	26.64 (15.47 - 37.81)	<.001	25.71 (14.11 - 37.30)	<.001	28.07 (17.02 - 39.12)	<.001
Class-wide	Difference (95%CI)	P	Difference (95%CI)	P	Difference (95%CI)	P
NRTI	23.44 (14.67 - 32.20)	<.001	24.09 (15.02 - 33.16)	<.001	25.27 (16.62 - 33.93)	<.001
NNRTI	-12.00 (-18.03 - -5.96)	<.001	-11.35 (-17.77 - -4.93)	<.001	-9.63 (-15.73 - -3.52)	0.002
PI (no minor)	6.05 (-0.15 - 12.26)	0.056	7.78 (1.22 - 14.34)	0.02	6.68 (0.44 - 12.91)	0.036
PI (minor)	14.89 (-5.96 - 35.75)	0.162	12.88 (-11.25 - 37.01)	0.295	13.75 (-9.21 - 36.72)	0.24
Individual DRM	Difference (95%CI)	Q	Difference (95%CI)	Q	Difference (95%CI)	Q
M184V	14.16 (8.34 - 19.98)	<.001	17.20 (11.05 - 23.36)	<.001	15.38 (9.55 - 21.21)	<.001
T215Y	7.61 (1.82 - 13.39)	0.131	8.64 (2.50 - 14.78)	0.099	7.85 (2.01 - 13.69)	0.124
D67N	8.96 (3.08 - 14.83)	0.059	9.05 (2.79 - 15.31)	0.091	9.56 (3.61 - 15.51)	0.065
L179D	-37.70 (-61.87 - -13.52)	0.059	-41.28 (-67.12 - -15.45)	0.051	-37.10 (-61.67 - -12.54)	0.073
L74V	-16.28 (-25.80 - -6.76)	0.032	-15.41 (-25.47 - -5.35)	0.064	-13.39 (-22.95 - -3.82)	0.103
V82A	13.81 (6.63 - 20.99)	0.01	16.81 (9.19 - 24.43)	<.001	14.96 (7.72 - 22.21)	0.003
I54V	10.69 (3.63 - 17.74)	0.059	12.35 (4.84 - 19.87)	0.05	10.97 (3.82 - 18.12)	0.073
Clusters	Difference (95%CI)	P	Difference (95%CI)	P	Difference (95%CI)	P
RT 1	4.86 (-1.45 - 11.17)	0.131	6.08 (-0.65 - 12.81)	0.076	5.77 (-0.62 - 12.16)	0.077
RT 2	3.01 (-3.40 - 9.42)	0.357	4.85 (-2.01 - 11.70)	0.166	4.93 (-1.58 - 11.44)	0.138
RT 3	9.14 (2.84 - 15.44)	0.004	8.09 (1.34 - 14.84)	0.019	8.27 (1.87 - 14.68)	0.011
RT 4	-2.41 (-8.96 - 4.13)	0.47	-1.46 (-8.40 - 5.47)	0.679	0.83 (-5.77 - 7.43)	0.806

RT 5	-10.16 (-16.76 - -3.57)	0.003	-8.81 (-15.84 - -1.77)	0.014	-7.64 (-14.34 - -0.95)	0.025
PI 1	10.19 (3.87 - 16.50)	0.002	12.96 (6.21 - 19.70)	<.001	11.04 (4.62 - 17.45)	<.001
PI 2	-0.22 (-6.99 - 6.54)	0.948	-0.38 (-7.53 - 6.77)	0.916	0.19 (-6.62 - 7.00)	0.956

1. Adjusted for age, hepatitis C, risk-group, subtype (1) and VL (2). 439 individuals were excluded due to missing values for subtype and risk group.

Table 5.12. Sensitivity 1: Additionally adjusting for type of drug received during the VF episode[1]

	Unadjusted		Adjusted (1)		Adjusted (2)	
	Difference (95%CI)	P	Difference (95%CI)	P	Difference (95%CI)	P
N=7,661						
Any	26.39 (13.65 - 39.13)	<.001	27.82 (16.82 - 38.81)	<.001	29.12 (18.75 - 39.49)	<.001
Class-wide	Difference (95%CI)	P	Difference (95%CI)	P	Difference (95%CI)	P
NRTI	25.45 (17.19 - 33.71)	<.001	26.17 (17.65 - 34.69)	<.001	26.45 (18.37 - 34.52)	<.001
NNRTI	-12.47 (-18.35 - -6.60)	<.001	-11.72 (-17.96 - -5.48)	<.001	-9.62 (-15.50 - -3.74)	0.001
PI (no minor)	5.51 (-0.52 - 11.53)	0.073	7.74 (1.38 - 14.11)	0.017	6.60 (0.60 - 12.59)	0.031
PI (minor)	12.27 (-7.92 - 32.47)	0.234	13.22 (-10.16 - 36.60)	0.268	13.63 (-8.42 - 35.68)	0.226
Individual DRM	Difference (95%CI)	Q	Difference (95%CI)	Q	Difference (95%CI)	Q
M184V	14.46 (8.82 - 20.11)	<.001	17.45 (11.49 - 23.41)	<.001	17.45 (11.49 - 23.41)	<.001
T215Y	9.39 (3.74 - 15.04)	0.022	10.63 (4.65 - 16.62)	0.012	9.57 (3.93 - 15.20)	0.023
D67N	10.53 (4.80 - 16.27)	0.013	11.22 (5.11 - 17.33)	0.012	11.57 (5.82 - 17.32)	0.003
L179D	-41.04 (-64.31 - -17.77)	0.016	-43.88 (-68.68 - -19.07)	0.012	-38.59 (-61.98 - -15.19)	0.024
L74V	-15.85 (-25.19 - -6.50)	0.021	-14.79 (-24.66 - -4.92)	0.056	-14.79 (-24.66 - -4.92)	0.052
V82A	14.51 (7.45 - 21.58)	0.003	17.79 (10.30 - 25.28)	<.001	16.37 (9.32 - 23.43)	<.001
I54V	11.00 (4.06 - 17.94)	0.032	12.90 (5.51 - 20.29)	0.012	11.70 (4.73 - 18.67)	0.023
Clusters	Difference (95%CI)	P	Difference (95%CI)	P	Difference (95%CI)	P
RT 1	5.90 (-0.29 - 12.09)	0.062	7.25 (0.65 - 13.84)	0.031	6.62 (0.42 - 12.82)	0.037
RT 2	3.42 (-2.84 - 9.69)	0.284	5.54 (-1.15 - 12.24)	0.105	5.34 (-0.96 - 11.64)	0.097
RT 3	9.28 (3.11 - 15.45)	0.003	8.97 (2.37 - 15.56)	0.008	9.04 (2.83 - 15.24)	0.004
RT 4	-3.32 (-9.70 - 3.07)	0.308	-2.25 (-9.00 - 4.51)	0.515	-0.39 (-6.75 - 5.98)	0.906

RT 5	-10.45 (-16.87 - -4.03)	0.001	-9.24 (-16.08 - -2.40)	0.008	-7.76 (-14.21 - -1.32)	0.018
PI 1	10.67 (4.47 - 16.87)	<.001	13.85 (7.24 - 20.46)	<.001	12.27 (6.04 - 18.51)	<.001
PI 2	0.13 (-6.40 - 6.66)	0.969	0.10 (-6.79 - 6.99)	0.977	-0.09 (-6.59 - 6.41)	0.979

1. *Adjusted for drug type, hepatitis C, risk-group, subtype (1) and VL (2). 439 individuals were excluded due to missing values for subtype and risk group.*
2. *Due to convergence issues, it was not possible to adjust for age, and VL had to be included as a categorical variable.*

5.5. Discussion

5.5.1. Detection of any resistance and CD4 declines

This chapter described the effect of drug resistance on CD4 decline among patients maintained on a failing treatment regimen. The first main finding is that CD4 counts declined less markedly during episodes where resistance any was detected compared to episodes with no resistance. The most likely explanation for this finding is that the comparator group (i.e. people in which no drug resistance was detected despite a situation of uncontrolled viremia during antiretroviral treatment) are individuals who are not adherent to therapy. As described in Chapter 1, drug resistance requires selective drug pressure in order to emerge. However, if patients are not taking any drugs the lack of drug pressure will mean that resistance is unable to emerge despite on-going viral replication. This is consistent with what has been found in other studies, where individuals who are not taking their ART were shown to experience both higher levels of VL replication and a more rapid CD4 decline (332). In addition to a more rapid CD4 decline, I also found that episodes without any detected resistance had higher starting viral load values, which provides further support for this hypothesis. Although the difference in CD4 decline according to the detection of drug resistance persisted in sensitivity analyses where I attempted to remove the effect of poor adherence, it is possible that the design of this sensitivity analysis only allowed me to exclude individuals who took intermittent treatment, but retained those patients who were taking no drugs at all. A validated measure of adherence or another more objective assessment of adherence, such as drug levels in plasma, would be required in order to confirm or refute the theory that a lack of detected resistance reflects poor adherence. Unfortunately, these are both currently missing in the EuroSIDA data.

Most previous studies have assessed the relationship between any drug resistance and a single CD4 measurement. For example, De Mendoza et al found an association between the detection of any resistance and higher CD4 counts in a cross-sectional analysis (602). In contrast, I did not find any evidence that CD4 counts at the start of failure episodes differed according to the detection of drug resistance. However, cross-sectional analyses assessing this question are hard to interpret due to the potential for reverse causality. Previous studies looking at the effect of any resistance on longitudinal CD4 count changes are not common, and in the literature review I could only identify one study that described the effect of any resistance on longitudinal CD4 trends specifically. This study, a longitudinal analysis by Dionisio et al of 16 patients from Italy, failed to show an association between the detection of any resistance and CD4 slopes (608), although the analysis is likely to be underpowered because of the small sample size.

5.5.2. The relationship between class-wide resistance, individual DRM and CD4 decline

In order to compare the relative effect of different types of resistance on CD4 decline, I separately evaluated resistance mutations associated with specific drug classes (NRTI, NNRTI and PI) as well as individual DRM after restricting to episodes for which at least one DRM was detected. This was done to potentially remove the allegedly poor adherent group in which no resistance was detected despite virological failure on treatment. The results from this analysis showed that CD4 counts appeared to decrease less steeply during episodes where NRTI resistance was detected. This is an interesting observation, and it is possible that these findings reflect differences in replication capacity associated with certain mutations that confer NRTI resistance (507). In addition, I found weak evidence indicating that CD4 counts declined less rapidly also during episodes where major PI mutations were detected, although the magnitude of this effect was not as large as for NRTI resistance. CD4 counts also declined less rapidly during episodes where major and minor PI resistance were detected, although these differences were not significant. In contrast, CD4 counts declined more steeply during episodes where NNRTI resistance was detected, consistent with reports that many NNRTI mutations have relatively high fitness.

Manual step-wise adjustment showed that adjustment for VL had very little effect on the effect estimates. The lack of impact of the VL adjustments on the estimates appear to indicate that the findings are not mediated through viral load. This is an interesting result and somewhat counterintuitive from a virological stand point. However, the presence of drug resistance has been associated with reduced rates of CD8+ T cell activation independent of the level of viral replication, which in turn is associated with a slower rate of CD4 decline (616). This indicates that it is possible that the presence of resistance could affect CD4 cell kinetics in beneficial ways that are not mediated through viral load (617). However, it is also possible that the adjustment for VL does not completely remove the effect mediated through VL. It has been shown that in many situations a model adjusting for a mediating variable does not accurately estimate the total direct effect of the exposure on the outcome, and in some cases spurious associations between the exposure and outcome can be introduced as a result of such adjustments (618–620). This should be born in mind when interpreting the results from the step-wise adjustments.

Regarding specific individual mutations, I found evidence to suggest that CD4 counts declined less markedly during episodes where the mutations M184V, T215Y, D67N in the reverse transcriptase and V82A and I54V in the protease were detected. The M184V mutation in the reverse transcriptase has been frequently shown to adversely impact replicative capacity

(595,621–623), and it has been suggested that preserving this mutation could result in a clinical benefit for patients (599,600). The size of the reduction in CD4 decline associated with the M184V mutation (17 cells/mm^3/year) was smaller than that associated with NRTI resistance overall (27 cells/mm^3/year). When restricting the analysis to episodes where the M184V mutation was not detected, the size of the NRTI effect reduced only marginally (24 cells/mm^3/year). This indicates that other NRTI mutations could also be influencing the speed of the CD4 decline. In my analysis, D67N and T215Y were both associated with moderately reduced CD4 declines of around 10 cells/mm^3/year. D67N has been shown to have relatively high RC (507,624), whereas the T215Y mutation has been associated by some researchers with an impaired RC when this mutation is present in a WT sequence background (623,624). However, these results are not consistent. Devereux et al found that the D67N mutation was associated with *in vitro* reduced fitness, ranging between a 10.5-24.7% reduction (623), whereas an analysis by Eastman et al from 1998 estimated that the relative fitness of the T215Y/F mutation following drug withdrawal could be as high as 98% compared to WT (625). Comparisons are further complicated by different laboratory methodologies as well as differences in the formulas used to calculate relative fitness estimates (626).

The V82A mutation has been reported to negatively impact the processing capacity of the PR which has been hypothesised to cause a lower replication capacity (627). This is in agreement with a small study determining the effect of PR mutations after interruption of therapy, which showed a 21-49.3% loss of fitness associated with the presence of the V82A mutation (326,626). However, other *in vivo* experiments have estimated that the fitness of V82A in the presence of Ritonaivr was as high as 96-98% compared to a WT virus (625). I54V, a nonpolymorphic mutation which is selected primarily by indinavir and lopinavir and reduces PI susceptibility when present in combination with V82A (394), has also been previously found to moderately reduce fitness (623), in support of my findings.

One NNRTI and one NRTIassociated mutations were weakly associated with steeper CD4 declines: V179D and L74V, although the evidence supporting an association with L74V disappeared upon adjustment for VL. V179D is a polymorphic NNRTI mutation, which can act in synergy with K103N to reduce Nevirapine and Efavirenz susceptibility (394). There is substantial evidence that viral variants with the K103N mutation have high fitness (507), and it is possible that the presence of V179D could act as a marker for the presence of K103N. In my analysis episodes in which K103N also showed steeper CD4 declines, although not significantly so after adjusting for multiple testing. However, the V179D mutation has been found to outgrow strains carrying the Y181C or V106A mutation and to only have a slight reduction in replication capacity compared to wild-type in an analyses by Archer et al., although the

researchers were unable to definitively establish the relative replication capacity of V179D due to insensitivity in the direct sequencing assay for that mutation (628).

The finding that CD4 declines are less steep during episodes where the M184V mutation was detected is in agreement with a very recent analysis by Hoffman et al. who analysed a large cohort of patients from South Africa and found that CD4 counts declined 11 cells/mm^3/year less rapidly during episodes of persistent viraemia during which M184V was detected compared to episodes during which it was not, although chance could not be ruled out as an explanation of this finding (p=0.1) (629). Other studies of resistance and CD4 decline have, as previously mentioned when describing the results from my literature review, been relatively small and found conflicting results. Antinori et al also found evidence that both M184V and V82A were associated with immunological recovery despite on-going viral replication in univariable analyses, although not in multivariable analyses adjusting for viral load (604). Nicastri et al found that the M184V mutation was more commonly detected among patients who experienced discordant virological-immunological responses than among patients who did not (603), which supports a potential immunological benefit of the presence of this mutation. These two papers did not present any insignificant multivariable results, and it is therefore difficult to assess whether adjustment for VL simply affected statistical significance or whether the magnitude of the effect was largely attenuated and close to a difference of zero, as the authors interpretation of the data would imply. The only trial investigating the effect of an M184V preserving strategy in the context of cART, COLATE, did not find any virological benefit or 3TC continuation (599). However, the patients in the trial are not representative of those of the population studied here as not many were people kept on failing regimens. Indeed, the vast majority of these patients received 3 or more active drugs in addition to 3TC, and by 48 weeks from the date of enrolment into the trial, around half of these patients had achieved a VL<50 on their new regimen. In contrast, the median GSS of the baseline regimens in my analysis was relatively low, at 1, and patients follow-up was censored if viral suppression was achieved. The conclusions reported by the COLATE investigators was that any benefit of a M184V preservation strategy may only be apparent among patients for whom a fully suppressive regimen cannot be constructed (599).

5.5.3. Clusters of mutations

I identified a total of seven clusters of mutations in this analysis, five in the RT and two in the PR region. Three of the RT clusters were relatively well-known, and closely resembled the well-described TAM mutations and the Q151M complex. I also identified two previously undescribed RT clusters. These broadly represented cross-NNRTI and ABC/DDI resistance and cross-NNRTI resistance respectively. The first PR cluster I identified represented broad PI

resistance, and contained a large number of both major and minor PI mutations. The second PR cluster was smaller, consisting of four minor PI mutations.

Other analyses have used a range of analytical techniques to study clustering of mutations. Wittkop et al also applied PCA to data from the ANRS cohort in order to derive scores summarising the correlation of mutations in the PR gene (630). Their analysis extracted two principal components. The first of these had heavy loadings from substitutions in position 10, 90, 46, 54, 82, 33, 70 and 71. The second PC did not have as many significant loadings, although substitutions in 20, 36, 35 and 69 contributed to the component. Interestingly, these corresponds reasonably well with the clusters I identified. All of the mutations found to contribute to the first PC in the ANRS analysis also contributed to the first PC extracted in this chapter, although my analysis captured contributions from a slightly larger number of mutations. The 2nd PC in the ANRS analysis captured the correlation of some minor PI mutations, 3 of which (20, 36 and 69) also contributed to the 2nd PR PC that I identified. Similar clusters in the protease gene have been described by other researchers as well (631,632). Wu et al calculated the binomial correlation coefficient for a number of PI mutations and used this to construct unrooted neighbour joining trees describing clusters in the PR gene (631). They found evidence of a cluster involving positions 35, 36, 20 and 62; as well as 54, 82, 24 and 46. Similarly, Kagan et al studied correlations between pairs of mutations, and found strong correlations between substitutions in position 54 and 82 (632). In their analysis, the positions which had the highest number of correlations were 10, 46, 71, 90, 20, 73, 82, 63, 84, and 54.

Mutations tend to cluster for a variety of reasons. Kagan et al conducted a structural analysis of the protease and found that most mutations with strong binary correlations were located within a relatively short physical distance from each other (632). This indicates that mutations which correlate strongly may have a degree of functional dependency, that is, interact directly with each other. However, their analysis also described correlations between some mutations located further away from each other, most notably for substitutions in position 46 relative to positon 90. This correlation is harder to explain, and the authors suggest that substitutions in position 46 may increase the catalytic activity of the protease, and therefore have a strong selective advantage irrespective of the location of other mutations (632). Further biochemical and structural analyses of the protease gene are needed in order to explain why and how these patterns of PI resistance emerge.

In terms of RT mutation clusters, Rhee et al studied correlations between mutations in both the RT and PR gene (633). Similar to the findings described here, the main clusters identified were TAM-1, TAM-2 and Q151M. These clusters have been relatively well described as they

confer complete cross-NRTI resistance (TAM-1 and TAM-2) or resistance to all NRTI's apart from TDF (151 complex) (335). The strongest correlation between an NRTI and NNRTI resistance mutation pair identified by Rhee et al was for substitutions in 74 and 181. This is in agreement with my analyses, where these two mutations contributed to the same component.

Despite the PCA analysis seeming to relatively accurately capture known clusters of mutations relatively accurately, the extracted components explained a low proportion of the overall variance in the dataset. This indicates that the variables included in the PCA may only be weakly correlated. However, other researchers who have applied PCA to HIV resistance genes in order to study clusters of mutations have found similarly low proportions of total variance explained (630,632,633).

5.5.4. The TAM-2 cluster and a cluster of broad PI resistance may be associated with reduced CD4 decline

Individuals with scores in the highest quartile of the 3rd RT cluster (TAM-2) experienced somewhat reduced CD4 declines, as did individuals with scores in the highest quartile of the 1st PI cluster, which represented broad PI resistance. Individuals with scores in the highest quartile of the 5th RT cluster, which included K103N, experienced steeper CD4 declines compared to individuals with lower scores on this component.

Although the TAM-2 mutations D67N, K70R and K219Q have relatively low fitness costs when present alone (507), the presence of 70R has been shown to result in a 6-fold reduction in fitness when it emerges in viruses already carrying D67N mutation , and a 9.4-fold reduction in fitness when present in viruses carrying both D67N and K219Q (634). My results are in agreement with this, and indicate that the presence of these mutations in combination may have an effect on CD4 decline. Such a combined effect may also explain why the detection of each of these 3 mutations individually resulted in less steep CD4 declines, although only significantly so for the D67N mutation after correcting for multiple testing. The impact of major PI resistance mutations on replication capacity is complex to predict as these mutations often appear in combination with several minor PI mutation that can restore viral fitness (507). However, it is possible that the 1st PI cluster could capture the effect of a number of major PI mutations which, when present in combination, could be associated with favourable CD4 changes. I also found weak evidence that the 5th RT cluster was associated with steeper CD4 declines. The mutations that formed part of this cluster include K103N and L100I, both of which are known to have a very small fitness cost or, in the case of L100I, has been shown have higher fitness than WT viruses (507). The lack of an association between the cluster representing the 151M resistance pattern agrees with previous research finding that this cluster does not seem to affect fitness (507).

5.5.5. CD4 decline and viral load thresholds

Although there was a general tendency for a better CD4 count recovery in people showing viral load suppression comparing to each individual estimate of maximum level of viral replication, it is worth noting that I couldn't find any clear VL threshold below which CD4 counts did not decline. This is in slight contrast with previous studies which have found that CD4 slopes tend to be less steep for lower viral loads (362,364,635), including a EuroSIDA analysis that studied CD4 counts trends among patients receiving cART (615). This analysis found that overall, CD4 counts remained stable at VL values between 500-10,000 copies/mL in the population. However, there was a significant interaction with type of cART regimen, with individuals on an NNRTI based regimen still experiencing significant CD4 count decreases in the 500-10,000 copies/mL strata , and the increase in CD4 counts at VLs below 10,000 copies/mL appeared to be restricted to individuals receiving a ritonavir-boosted PI (615). The use of PI's in my analysis was relatively high (60%), but only 76 individuals received a ritonavir-boosted PI (1%), and this difference in treatment regimens used, mostly as a consequence of the early calendar years included in my analysis, might explain the discrepancies. The PLATO cohort, which has previously reported increasing CD4 counts for individuals with a VL<10,000 copies/mL, was designed to only include individuals with triple-class resistance (364). The PLATO study population is therefore relatively different compared to the one described here, as I did not require individuals to have detected resistance in order for them to be included. A recent analysis of patients failing first line ART in low income settings, which had a similar design and analytical approach to my analysis, was also unable to identify a clear VL threshold above 500 copies/ml below which CD4 counts remained stable (636). Nonetheless, it is worth noting that CD4 decline in this analysis was less marked among individuals who had a viral load supressed below the mean of their pre-ART VL levels, and higher among those individuals who had a VL>100,000 copies/mL.

5.5.6. Limitations and strengths

There are several important limitations to this analysis. Firstly, as different mutations can modulate the fitness effects of other mutations (634), the pattern of resistance is likely to reflect the fitness of a given strain more accurately than the presence of absence of individual mutations. I attempted to study the combined effect of mutations on CD4 decline through PCA, but other techniques, such as fitness landscapes (637), may capture such interactions more accurately. Secondly, I did not have a validated measure of adherence available in either UK CHIC or EuroSIDA. Adherence could influence the results as it is likely to influence both the presence of resistance and CD4 decline (638). A validated measure of adherence would also have been required in order to verify the hypothesis that people with no detected resistance are those with the poorest adherence. Power and limited data may also have been an issue.

Despite using combined data from two large cohorts, the relatively low prevalence of individual mutations means that some of the estimates, particularly for rare mutations, suffer from low precision. If appropriate data sources are identified, it would be of value to repeat these analyses in larger cohorts in order to derive more precise effect estimates. I was also unable to assess the impact of mutations or mutational patterns in gene regions apart from the RT and PR. It would have been of interest to study the effect of mutations in the envelope or integrase genes (507). This would be of particular interest as it has been reported that mutations in *gag* can modulate the fitness of PR resistant viruses (639,640). A further limitation is the lack of resistance testing for some patients leading to the potential for selection bias. As shown in Chapter 3, not all individuals who experience VF are tested for resistance, and these individuals differ in important ways from those that are tested for resistance. This could limit the generalisability of my findings.

As previously stated, the median follow-up contributed by patients in this study was relatively low. It is possible that a virus which initially harbours fitness-compromising mutations develop compensatory mutations that restore fitness over time. A 2006 study by Machouf et al found that as mutations accumulated the presence of resistance stopped having a beneficial effect on VL levels, and instead seemed to cause steeper VL declines as compared to WT strains (641). This conclusion is echoed by Barbour et al in a 2002 analyses of CD4 count trends among patients maintained on a failing PI regimen (609). Although the authors found relatively durable benefits from therapy maintenance, there were a number of patients who nonetheless experienced decreasing CD4 trends. This indicates that any benefit derived from being maintained on a failing regimen is likely to reduce over time as the viruses accumulate compensatory mutations (609,641). Although it was not possible to restrict this analysis to individuals contributing longer FU due to small numbers, it is important to take this likely limitation into account when interpreting the results.

In terms of strengths, the main strength of this analysis is the large sample size. Using data from both EuroSIDA and UK CHIC allowed me to include over 5,000 individuals, making this the largest analysis investigating this question to date. This allowed me to assess the effect of a range of individual DRM on CD4 trends in addition to describing the effect of any and class-wide resistance. In contrast to the majority of previously published studies I was also able to describe CD4 count changes over time rather than at a single time-point. The analytical approach, including correcting for repeated measures and multiple testing, also represents a strength in comparison to previous research.

5.5.7. Conclusions

Bearing these limitations in mind, our findings provide some support for the hypothesis that CD4 decline may be less marked when individuals experiencing VF are maintained on regimens that preserve specific mutations associated with reduced viral fitness. This could have implications for individuals in low income settings who have failed all existing available lines of ART, and suggests that in these instances the inclusion of lamivudine/emtricitabine and a boosted PI may be the best option in terms of composing a salvage regimen. However, the likely effect of the presence of a particular mutation on annual CD4 decline is likely to be complex to predict due to the presence of epistatic interactions and potentially confounding effects which are hard to measure, such as residual drug action and adherence. Further research is needed to evaluate the clinical benefits of specific treatment strategies that aim to preserve particular resistance patterns among individuals for whom a fully suppressive ART regimen cannot be prescribed, as well as to elucidate the mechanisms through which any beneficial effect is mediated. Until then, efforts should focus on ensuring that those who experience VF have access to individualised care and a broad range of antiretroviral drugs, and, where possible, novel ART drugs through compassionate early access programs. The clinical implications of the findings and suggestions for further studies are discussed in more detail in Chapter 8.

5.5.8. Dissemination of results

These findings were presented at the Glasgow HIV Therapy conference in 2014 (Appendix VIII). A paper based on the analyses is under review with Antiviral Therapy.

Chapter 6 . Rate of accumulation of drug resistance mutations during virological failure according to the level of viral replication

6.1. Introduction and Objectives

In the previous chapter I showed that the detection of certain resistance mutations among individuals kept on a failing treatment regimen may have a small but significant positive effect on CD4 cell count trends. Although it is always preferable to switch to a fully suppressive regimen if such a regimen is available (642), there are nonetheless some situations which result in individuals being maintained on failing treatment regimens. Firstly, among patients who have extensive and complex resistance patterns, there may be no option to switch to a fully suppressive regimen even in high income settings. Secondly, in low income settings, it is recommended that individuals have adherence interventions and a confirmatory viral load (VL) measurement taken before a switch in therapy occurs. Due to programmatic delays this means that individuals may be maintained on a regimen to which they have developed resistance for long periods of time (72,643). The WHO also recommends routine viral load monitoring, but as this is not available in all settings clinical and immunological criteria are still used to determine when a person should switch treatment (72,629). This can further delay treatment switches as CD4 counts can remain high despite ongoing viral replication and the presence of resistance (629). There may also be a reluctance among clinicians to switch patients until symptoms appear, in order to preserve further treatment lines for as long as possible. Finally, clinicians in low income settings may not be able to switch patients failing second line therapy in a timely manner as access to third or higher line regimens is generally limited (586). This could affect a potentially large number of people. In a 2010 multi-cohort analysis, Pujades-Rodriguez and colleagues estimated that 18.8% of individuals receiving second line therapy in a variety of low income settings met WHO failure criteria after a median of 11.9 months following the start of second line therapy, meaning they would need access to third line regimens (587). Taken together this means that despite the large availability of different drugs in high income settings, globally, there is still a proportion of people living with HIV who are maintained on failing treatment regimens.

One of the primary concerns when maintaining individuals on failing treatment regimens is the accumulation of resistance mutations which may compromise subsequent lines of treatment once they become available and contribute to the spread of transmitted resistance (422,644).

Estimates of resistance accumulation are relatively rare, as it requires repeated resistance testing (356,645–647). The rate of resistance accumulation is also influenced by a number of different factors, including adherence, the type of treatment used, the fitness impact of specific mutations and the level of viral replication (417,648). However, despite its importance, the relationship between viral replication and resistance accumulation in routine clinical care has been poorly defined. Higher levels of viral replication should, in theory, lead to a higher number of replication errors and consequently an increased number of resistance-conferring mutations occurring. However, while some authors have found this to be the case (336,355), others describe an inverse U-shaped relationship between viral load levels and resistance detection, where the probability of detecting resistance is the highest at intermediate levels and lowest at the extremes (565,567,649). Others have found no relationship between VL levels and the development of resistance (500,650,651).

Differences in study design and in the timing and categorisation of the VL exposure variable is likely to at least partly explain these differences. Several different study designs can be, and have been, used to study the relationship between viral replication and drug resistance using observational data. First of all, the prevalence of resistance can be described according to the viral load at the time the resistance test was done (336,486,565). This simple, cross-sectional approach can generate useful information for clinicians looking to determine the chance of detecting resistance when doing a test, but interpretation of the association between the viral load and resistance detection is difficult due to the potential of reverse causality as only a single time-point is studied. This means that it is not clear whether a particular mutation appeared and consequently affected the VL, or whether the VL levels affected the appearance of mutations. As tests done at high VLs are more likely to amplify and consequently have results recorded, it is also possible that any observed relationship simply reflects limitations in the genotyping technology. Alternatively, the prevalence of resistance can also be described according to a viral load measure taken before the test was done: for example, at the first raised VL following suppression or at the start of a certain treatment regimen (652). Again, this generates useful information for clinicians looking to determine the consequent risk of resistance development given the viral load observed at a key previous point of the person's care. However, resistance testing is typically infrequent and it is possible that any detected resistance was already present at the time the viral load measurement of interest was done. The final option involves using repeated resistance tests, and calculating the rate of accumulation of new resistance mutations (356). This is a better approach as it allows knowing which new mutations have been acquired, although the timing of the appearance of resistance mutations is still interval censored.

In this chapter, I used the latter approach and aim to describe how the rate of resistance accumulation is affected by well characterised a priori definitions of viral exposure when individuals are maintained on a failing treatment regimen. The specific objectives were to:

1) Estimate the rate of accumulation of any and specific resistance mutations while individuals are maintained on a failing treatment regimen

2) Investigate how the rate of accumulation of any resistance varies according to a number of exposure variables designed to capture different aspects of patients' history of viral replication:

    a. Viral load levels at the start of an episode of virological failure (VF) (defined below)
    b. Average VL during a VF episode
    c. Peak VL during a VF episode
    d. Estimated VL slope during a VF episode
    e. Copy-years viraeamia, a measure of cumulative VL exposure over the total duration of the failure episode

6.2. Literature Review

I expanded the literature review done in Chapter 3 to include papers from non-European settings, added the search terms "accumulation" and "rate" and selected any papers that reported on the relationship between drug resistance and viral load levels. To narrow the search, I excluded studies which reported only on a specific mutation or resistance to a specific drug, but included those studying a class of resistance. I further excluded those studies which only focused on restricted populations (eg, prisoners) as they are likely to not be comparable with the population in the EuroSIDA cohort, which was used for this analysis. The search results are shown in Figure 6.1 below, and the data extracted from the studies summarised in Table 6.1.

Figure 6.1. Identification of articles for inclusion in the literature review

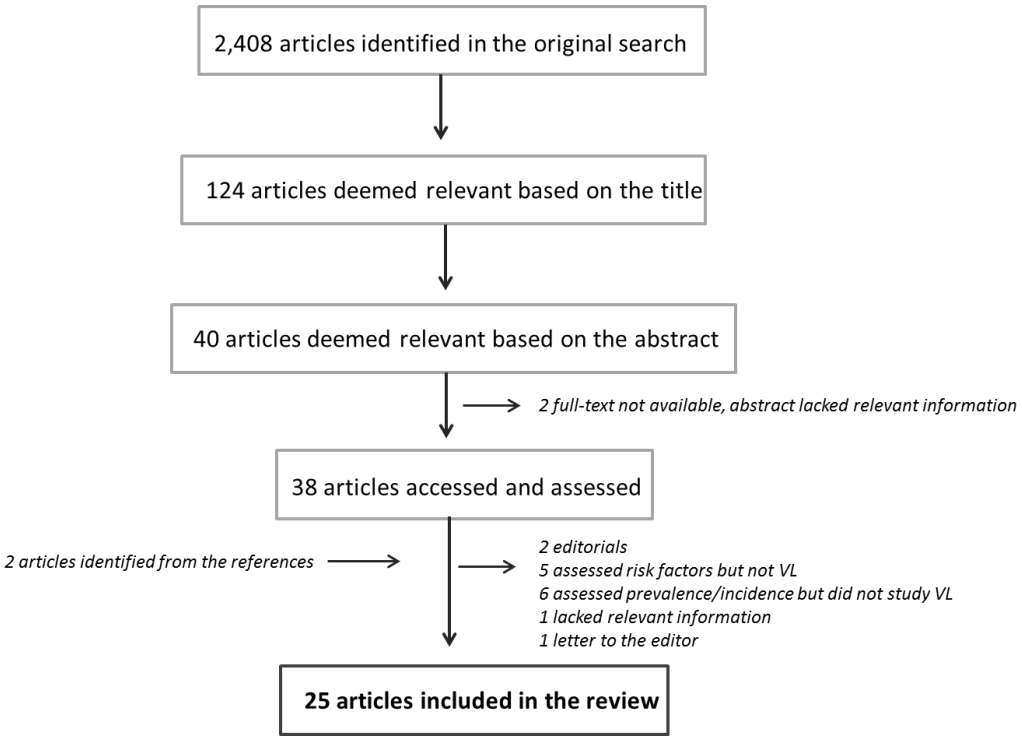


Table 6.1. Results from the literature review							
Author	**Year**	**Data Source**	**N**	**Study Design**	**VF definition and timing of VL measurement**	**Relationship between VL and resistance**	**Ref**
Pinoges et al	2015	Electronic health data from MSF clinics in Malawi, Kenya, Uganda and Cambodia	2,257	Cross-sectional	VF was defined as a VL> 400 (limit of detection). VL was measured at the time of the genotypic test. VL was not categorised.	Not assessed in multivariable models. The median VL was higher among individuals who had resistance to TAM's compared those with no TAM's (73,785 vs. 13,806 copies/mL; *P* = 0.001)	(653)
Santoro et al	2014	Retrospectively analysed plasma samples from patients in central Italy	2,200	Cross-sectional	VF was defined as a VL>50. VL was measured at the time of the genotypic test. The VL strata considered were 50-200, 201-500, 501-1000, 1001-10000, 10001-100000 and >100000.	The prevalence of resistance varied significantly by viremia strata (p<0.001), and was characterized be a bell-shaped curve. The prevalence according to increasing VL categories were: 53, 70, 74, 86, 77 and 63 % respectively. The highest prevalence was found at the 1001-100000 category (86%). Multivariable analyses were not performed.	(567)
Franzetti et al	2014	Cohort study in Italy (ARCA cohort), 2003-2012	5,246	Cross-sectional	VF was defined as a VL > 200 copies/mL after at least 6 months of ongoing treatment. VL was measured at the time of the genotypic test. The categories considered were <4 log_{10} copies/mL, 4-5 log_{10} copies/mL and >5 copies/mL.	The odds of detecting resistance to either PI, NRTI or NNRTI was higher in lower VL categories. Although a linear trend was not evaluated, the odds ratios appeared to indicate that the odds of detecting resistance was consistently the highest in the lowest VL category (<4 log 10 copies/mL; aOR=3.06 [2.59-3.61]; 2.61 [1.89-3.60] and 1.70 [1.40-2.07 for NRTI, NNRTI and PI resistance respectively) as compared to the highest.	(486)
Assoumou et al	2013	The ANRS laboratory network in France	506	Repeated cross-sectional	VF was defined as a VL>50 after at least 6 months of cART. VL was measured at the time of genotyping. The VL strata considered were 50-500, 501-1000 and > 1000.	The frequency of resistance increased with plasma viral load: 49% 51-500, 65% 501-1,000 and 67% in >1000. Multivariable analyses were not conducted.	(488)

De Luca et al	2013	The SEHERE collaboration in CHAIN	20323	Repeated cross-sectional	VF was not defined; patients included had at least 90 days of treatment and a genotype available. VL was measured at the time of genotyping. VL was modelled linearly.	VL was not linearly associated with the detection of any resistance (aOR=1.0, 95%CI=1.0-1.1), although a higher VL was associated with decreased odds of detecting NRTI resistance (aOR=0.95, 95%CI=0.9-1.0) and increased odds of detecting NNRTI (aOR=1.1, 95%CI=1.1-1.1) and PI (aOR=1.1, 95%CI=1.1-1.2) resistance.	(336)
Liégeois et al	2012	WHO survey of clinics in Gabon	375	Cross-sectional	VF was defined as a VL>300 while on ART. VL was measured at the time of genotyping among those with a VL>1,000. VL was modelled linearly.	VL at the time of genotyping was not linearly associated with the detection of drug resistance mutations (DRM) (p=0.75) in univariable analyses. Multivariable analyses with VL were not conducted, effect sizes were not presented.	(650)
Li et al	2012	University of California, San Francisco, SCOPE cohort	47	Cross-sectional	VF was defined as a detectable VL above the detection limit of the assay but below 1,000 copies/mL. VL was measured at the time of genotyping. Details of how VL was modelled not published.	No association between VL and the detection of DRM was reported.	(651)
Praparattanapan et al	2011	Retrospective analysis from a clinic in Chiang Mai, Thailand	59	Cross-sectional	VF was defined as receiving NNRTI-based ART for at least 6 months, having achieved an undetectable VL and consequently having another VL>1000. The evaluated VL was measured at the same time as the GRT.	Compared the probability of detecting a range of individual mutations: The only difference found was that the detection of 3-5 NRTI mutations was more likely to be detected at VL>4 log (p=0.001). Effect sizes were not published.	(654)
Cozzi-Lepri et al	2011	Analysis from the EuroSIDA cohort	227	Longitudinal, repeated resistance tests	VF was defined as having one VL>500 after at least 6 months of receiving an NNRTI regimen while still receiving an NNRTI regimen. The VL evaluated was measured at the start of the VF episode, with GRT's occurring at a later time-point. VL	A 1 log increase in the VL at start of the VF episode was associated with a lower risk of consequently detecting NNRTI resistance (adjusted RR=0.8, 95%CI=0.64-1.01, p=0.06).	(647)

Sigaloff et al	2011	Analysis from the PASER-M network at 13 sites in 6 African countries	250	Cross-sectional	VF was evaluated retrospectively after a switch to second line therapy had occurred. VL was measured at the same time as the genotyping. VL was modelled linearly.	VL was borderline linearly associated with the detection of NRTI cross-resistance (aOR= 1.57, 95%CI=1.00 to 2.47), but not with the detection of TAM's (uOR=1.09, 95%CI=0.73 to 1.60).	(655)
Prosperi et al	2011	Analysis of the SEHERE database	11492	Cross-sectional	VF was not considered an inclusion criteria. Patients with resistance tests taken after at least 6 months of uninterrupted ART were included. VL was measured at the time of the genotypic test. The categories considered were <50; 51-200; 201-500; 501-1000; 1001-10000; 10001-100,000 and <100,000.	VL strata was associated with the detection resistance (all p<0.0001) . Resistance increased at higher VL levels, peaking in the 1001-10000 copies stratum (*reference*). Higher stratum than this was followed by a decrease (OR=0.4, 95%CI=0.34–0.46 amongst those with VL>100,000 copies/mL). It was lowest at VL<50 copies/mL (OR=0.14, 95%CI=0.11–0.17).	(649)
Bannister et al	2011	Analysis of the EuroSIDA database using multiple imputation	6498	Cross-sectional	VF was not considered an inclusion criteria. individuals were required to have started ART and have a VL measured within 3 months before the GRT.	Estimated resistance prevalence was lowest when the current VL was <50 copies/mL, although this was not formally evaluated; effect estimates were not published.	(473)
Lima et al	2010	The HAART observational medical evaluation and research cohort in British Colombia, Canada	1820	Longitudinal, no repeated resistance tests	VF was not considered an inclusion criteria. Individuals were required to start HAART between 2000 and 2007. VL was measured throughout the FU period, and categorised as "change in VL" from the start of treatment until the emergence of resistance or censoring, whichever came first.	Change in VL was significantly associated with the emergence of resistance (p<0.001). The lowest odds or resistance development was found among individuals with a VL change of <-2 $\log_{10}$ copies/mL over the total FU (*reference),* ie, a VL decrease. The odds of detecting resistance was higher at VL decreases that were not that marked, but not higher at VL increases (aOR=1.23, 95%CI=0.50-3.03)	(656)
Mackie et al	2010	The UK HIV Drug Resistance Database	3791	Cross-sectional	VF was not considered an inclusion criteria. Individuals were required to have started ART and have a VL within -4 to +2 weeks of the resistance test. VL was categorised into strata: <300, 300-999, 1000-2999, 3000-9999, 10,000-29,999, 30,000-99,999 and >100,000.	Detection of resistance was most frequent among tests performed at VL of 300-10,000 copies/mL (*reference*) and decreased above and below these levels. It was highest at VL>100,000 (RR=0.69, 95%CI=0.65–0.74) Trend the same irrespective of the type of resistance.	(565)

Hanson et al	2009	Cote d'Ivore national drug access program	645	Longitudinal, no repeated resistance tests	VF was not considered an inclusion criteria. All individuals with >300 days of FU after ART initiation and at least 2 clinic visits were included. Each patient had VL measured on 3-monthly visits after the start of ART. If VL was above 1000 copies/mL, a resistance test was done. VL was modelled at baseline (start of ART) and as change in VL from the start of ART: baseline VL in categories of <10,000; 10,000-100,000; >100,000 and missing and VL change as <0.5; 0.5-2, 2-4 and >4 log_{10} copies/mL.	Both baseline VL and change in VL was associated with the development of resistance in multivariable models. In terms of baseline resistance, the risk of detecting resistance decreased with increasing VL values, and was the lowest when baseline VL was >100,000 (aHR=0.30, 95%CI=0.16, 0.57). Considering change in VL, the risk of detecting resistance was the lowest when the change in VL was the smallest: with the lowest risk of detecting resistance found among those with a <0.5 log/10 copies/mL change in VL over the study period (aHR=0.12, 95%CI=0.05, 0.27)	(652)
Cozzi-Lepri et al	2007	Analysis of EuroSIDA data	110	Longitudinal, repeated resistance tests	VF was defined as a VL>400 copies/mL. Repeated resistance tests were required, and VL had to be raised from the first until the 2nd test. VL was measured throughout FU, and the difference in VL between the two tests was evaluated linearly for association with resistance accumulation.	A suggestion of an association change in viral load between t0 and t1 and the rate of accumulation of IASDRM [+0.35 (95% CI, –0.12 to 0.83) mutations per log_{10} larger increase in viral load; p=0.14.	(356)
Tozzi et al	2006	Analysis of data from a clinic in Rome, Italy	602	Cross-sectional	VF was defined as a failure to achieve virological suppression, or of at least two VL >1,000. VL was measured at the GRT. VL was modelled linearly.	There was no linear relationship between VL and the probability of developing NRTI (p=0.25), NNRTI (p=0.90) or triple class resistance (TCR; p=0.67). There was a borderline (p=0.048) association between pVL and the odds of developing PI resistance (aOR=0.68, 95%CI-0.61=1.00) for each log increase in the VL.	

Napravnik et al	2005	Analysis of data from a clinic at the university of North Carolina	98	Longitudinal, repeated resistance tests	VF was not considered as an inclusion criteria. Individuals were required to have two resistance tests taken more than 30 days apart. VL was measured during the FU period (1st to 2nd resistance test). Several measures of VL was used: peak, baseline, average and change in VL. All VL measures were evaluated in several ways: categorical, linear and using quadratic splines.	The average VL level between the two tests was associated with the development of resistance, with the highest risk of resistance development having been found among those with 3-4 log VL (aRR=2.11, 95%CI=1.43–3.09), and lower risk of resistance development among those with a VL below 3 or above 4. The change in VL between the two tests was also associated with resistance development. Those who experienced an increase in the VL > 0.2 cp/ml were at a greater risk of developing resistance (aRR=1.93, 95%CI=1.23–3.01). The other measures of VL were associated with the risk of developing resistance in univariable analysis but not after adjustment: No effect sizes or formal comparisons were presented.	(646)
Harrigan et al	2005	Analysis of the HOMER cohort in Canada	1191	Longitudinal, did not consider repeated resistance test	VF was not considered as an inclusion criteria. Individuals were required to start ART between 1996-1999. VF was measured throughout FU; resistance tests were ordered for VL measures with a VL >1000 copies/mL.VL at the start of ART was modelled linearly.	VL at the start of the VF epsiode was linearly associated with the detection of DRM both in univariable and in multivariable analyses, with higher VL associated with a higher risk of detecting resistance (aHR=1.59, 95%CI=1.29–1.96; p<.001)	(355)
Phillips et al	2005	Cohort study in the UK (UK CHIC and UK HIVDRB), 1998-2005	7891	Longitudinal, did not consider repeated resistance tests	VF was not considered an inclusion criteria. Individuals were required to start ART for the first time after 1996. VL at the start of ART was categorised into <100,000, >100,000 and missing.	A VL of >100,000 copies/mL at the start of ART led to an increased risk of resistance detection (aHR=1.36, 95%CI1.12–1.65)	(501)

Bangsberg et al	2003	The REACH cohort in San Fransisco	148	Longitudinal, repeated resistance tests	VF was defined as a VL>50 copies/mL. Individuals were genotyped twice during a period of VF while on stable ART to estimate the rate of resistance development. VL at the start of ART was evaluated linearly on the log scale.	VL at the start of the episode was not associated with the development of DRM (aRR=0.0846, p=0.61)	(657)
Kantor et al	2004	Stanford University Hospital	106	Longitudinal, repeated resistance tests	VF was not considered an inclusion criteria, although individuals were required to have two resistance tests while maintained on the same treatment regimen. Changes in the VL between the two tests was evaluated.	No difference in the development of new mutations among patients with rising or declining VL levels, effect sizes or p-values were not reported.	(357)
Richman et al	2004	Cross-sectional analysis of cohort study in the US (HCSUS study), 1998	101100	Cross-sectional	VF was defined >500 copies/mL. VL was measured at the time of the GRT, and categorised into two categories: 500-30,000 and >300,000.	Individuals with VL in the lower category had higher categories of resistance (OR, 2.91; 95% CI, 1.93–4.39)	(476)
Adjé-Touré	2003	Analysis of data from the UNAIDS drug access initiative in Abidjan, Cote d'Ivore	86	Longitudinal, did not consider repeated resistance tests	VF was not considered an inclusion criteria. Individuals were required to have been on ART for at least 6 months. VL was measured throughout FU, and VL at the start of a VF episode and change in VL from the start of ART was evaluated for its association with DRM linearly.	VL at the start of ART was not linearly associated with the detection of resistance (aOR=0.6, 95%CI=0.2–2.0), although virological response was strongly associated with the detection of resistance (aOR= 2.8, 95%CI=1.3–5.9) The lower the maximal virological response, the higher the odds of detecting resistance	(658)

In total, 25 articles that described the relationship between VL and drug resistance were included. The majority of these used a cross-sectional approach to study risk factors for detecting drug resistance, although a reasonable number (N=8) were longitudinal. A variety of viral load measures were used, the most common approach was to evaluate the relationship between resistance and VL at the time of the genotypic resistance test (GRT) (13/25 studies). Overall, the results were conflicting, and appeared to vary according to the classification of the VL measurement, the type of resistance present as well as the comparator arm used.

A number of authors that studied the relationship between the VL and the detection of resistance in a cross-sectional manner reported on an inverse U-shaped relationship, whereby the risk of detecting resistance was highest at intermediate VL levels and decreased both at low and high VL levels (565,567,649). Santoro et al, who conducted a large retrospective analysis of a clinical database in Italy, found a higher prevalence of resistance at VL levels between 1,000-10,000 copies/mL and lower prevalence of resistance at lower and higher viral loads (567). Mackie et al, described a similar trend using data from the UK HIV drug resistance database (565), where the highest prevalence of resistance was found among VL levels between 3000-10,000 copies/mL. An analysis of the SEHERE database in chain also confirmed a U-shaped relationship between resistance and VL, with resistance prevalence peaking at VL levels between 1,000 and 10,000 copies/mL (649). There are some suggestions that at VL levels below 1,000 the prevalence of resistance may increase in a more linear fashion (488), although most analyses have included too few resistance tests conducted at low viral loads to comprehensively confirm this (565). The authors of these studies suggest that the bell-shaped relationship between resistance and VL at the time of genotyping is likely to at least in part reflect adherence, with very high VL levels potentially being indicative of poor adherence (565,649). Poor adherence would allow both high viral replication and remove any selective pressure from the virus, thus resulting in a lack of resistance.

Among those modelling VL as a continuous variable, the majority of studies failed to find a linear association between VL at the time of genotyping and the detection of any resistance (336,500,650,651). However, Richman et al, analysing the large HCSUS cohort from the US, found some evidence that the lower the current VL the higher the odds of detecting resistance (476). This is in contrast with an analysis by Sigaloff et al of the PASER-M data from several countries in Africa, which found a linear association between VL levels and detection of cross-NNRTI resistance: the higher the VL the greater the odds of detecting cross-NRTI resistance (655). If the true relationship between resistance and current VL follows an inverse U-shape, it is possible that including VL simply as a linear covariate may have led to a misspecification of the model which could explain these seemingly contradictory findings. However, it is also

possible that the relationship between VL and resistance could vary according to the class of resistance detected, as the findings by De Luca et al suggests (336). In an analysis of the SEHERE collaboration in the CHAIN study primarily focused on time-trends in resistance, De Luca and colleagues find that VL at the time of genotyping is associated with decreased odds of detecting NRTI resistance and increased odds of detecting NNRTI and PI resistance (336). However, they found no evidence for a linear association between VL and the detection of any resistance. This suggests that both the class of drug the virus is resistant to and the statistical categorisation of the VL variable could influence the results found.

Out of the studies using a longitudinal design, the most detailed investigation into the relationship between viral load and resistance development was conducted by Napravnik et al in 2005 (646). They used data from 98 patients from a clinic in North Carolina, USA, and studied the rate of resistance accumulation among patients with at least two GRT's taken more than 30 days apart. They considered how several different measures of the VL affected the risk of accumulation of new resistant mutations detectable in majority virus. After adjustment for pre-specified confounders (final model specifications were not included in the paper), VL at the start of the VF episode and peak VL was found to not be associated with resistance despite considering these variables both as categorical, linear and using more sophisticated modelling using splines. However, the average VL level between the two tests was associated with the development of resistance. The highest risk of resistance development was found among those with intermediate (3-4 $\log_{10}$ copies/ml) average VLs. The change in VL between the two tests was also associated with resistance development, and individuals who experienced a greater increase in the VL were found to be at a greater risk for developing resistance (646). These results are in slight contrast to a similar study by Kantor et al, who also characterised the rate of resistance development among patients with repeated GRT's accessing care at Stanford University Hospital. With a sample size of 106 patients, they could not identify any difference in the rate of accumulation of new resistance mutations according to the VL trend between the two tests (357). However, two other studies employing a similar study design have found that the level of change in the VL between two resistance tests influenced the rate of development of resistance (356,656). Only one studied assessed a combined measure of VL levels and the duration of exposure to a certain VL level (658). Specifically, in an analysis of patients from Abidjan, Cote d'Ivore, Adje-Toure et al found that maximum virological response, which they defined as the difference between two VL measurements divided by the time between them, was as strong predictor of resistance development. The authors also found that VL at the start of therapy was not a predictor of resistance development after adjustment for maximum virological response (658).

6.2.1.What this analysis adds

A relatively large number of studies have reported on the relationship between VL levels and drug resistance, although most have done this as a secondary aim and did not consider different ways of modelling the VL. Relatively few studies reported on the rate of accumulation of drug resistance taking resistance patterns at the start of the VF episode into account, and only two small studies reported on the accumulation rate in relation to measures of viral exposure. A description of how new resistance accumulates given different levels of viral replication can provide important information for clinicians evaluating the implications of maintaining a patient on a failing treatment regimen, particularly in contexts where resistance testing is not available. Given the paucity of published studies assessing this comprehensively there is a gap in the literature on the relationship between the VL and the risk of resistance development. EuroSIDA is an ideal cohort to analyse this question due to the relatively large availability of repeat resistance tests, arising primarily from retrospective genotyping done on prospectively stored plasma samples.

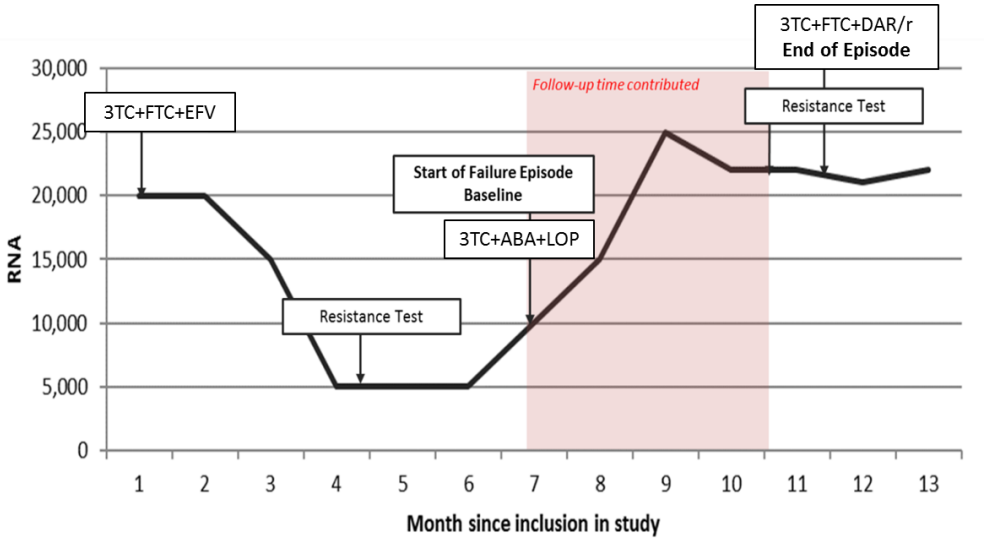
6.3. Methods

6.3.1. Inclusion Criteria

Persons from the EuroSIDA cohort, database version D42, who experienced VF to a cART regimen during prospective FU with at least one resistance test available during this VF episode were included. VF was defined in using a similar approach to that used in Chapter 5, but a lower VL cut-off was used as I was interested in also characterising resistance development also at lower viral loads. VF was therefore defined as two or more consecutive measures of VL >50 copies/ml while the individual was kept on the same ART regimen, in individuals who had been on ART for at least 6 months at the start of the VF episode. This was defined as the first VL measurement >50 copies/mL of two consecutive > 50 copies/mL, and the end date as the first VL measurement below 50 copies/mL or at the point where the ART regimen was changed (defined as either stopping or adding one or more drugs to the regimen). Individuals were also required to either be experiencing their first VF from naive or have a resistance test available no more than 3 months before the start date of their VF episode. This allowed us to estimate the level of resistance at the start of episodes.

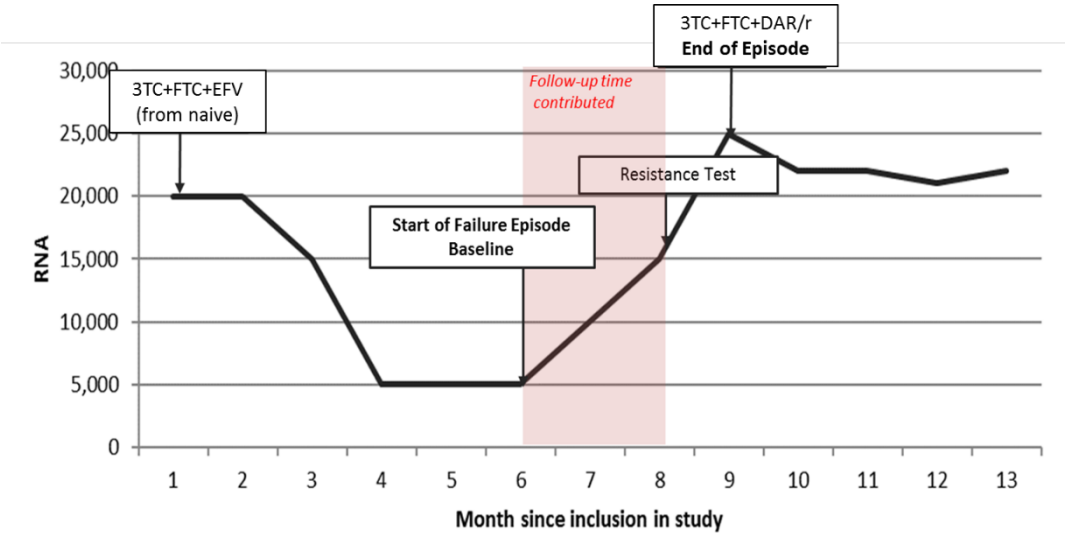
Figure 6.2. Hypothetical data illustrating the inclusion process

(a)



This individual started treatment at 1 month since inclusion in the study, but did not fully suppress. A resistance test was ordered after 4.5 months, and the individual swapped to 3TC+FTC+LOP. This was classed as the start of VF as it was the first of two consecutive VL measures above 50 copies/mL on this regimen and it was more than six months since initiation of a new regimen. Another resistance test was done after 10.5 months and the regimen swapped after 11.5 months. The FU time contributed ranged from the start of the VF episode until the second resistance test.

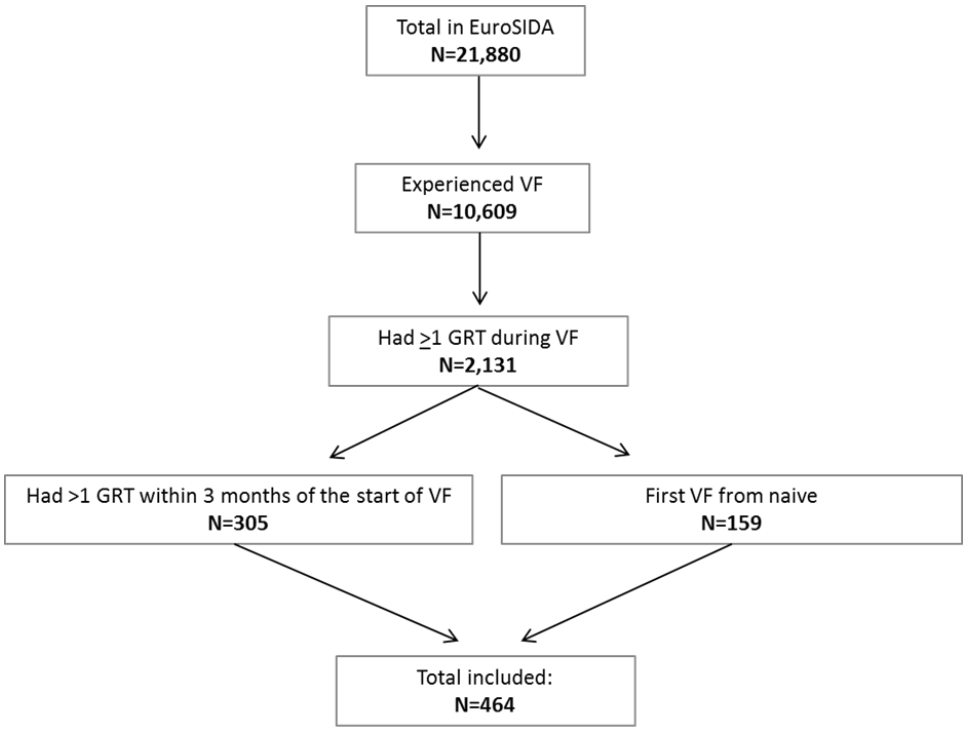
(b)



This individual started ART from naïve, and experienced VF after 6 months of not suppressing, when he had a VL measurement above 50 copies/mL. A Resistance test was done at 8 months since ART initiation. As this was the first regimen given from naïve, no resistance test was required before the start of the FU time and the individuals was assumed to start the VF episode at the time of the first eligible VL measurement. The person contributed FU time until their resistance test.

Individuals could contribute more than one failure episode, and more than one resistance test during a single failure episode. A flowchart of the selection process is shown below in Figure 6.3.

Figure 6.3. Selection flowchart



6.3.2. Viral load replication exposures

Based on the results from the literature review, it was decided to evaluate five different exposure measures for the VL:

1. VL at the start of the VF episode
2. Peak VL, defined as the highest VL measurement taken between start of the VF episode and the last resistance test during a VF episode
3. Average VL, calculated as the mean of the VL measurements taken between the start of the episode and the last resistance test during a VF episode
4. VL trajectory, the estimated VL slope between the start of the episode and the last resistance test during a VF episode
5. Copy Years Viraemia (VCY), calculated using the trapezoidal approximation described in detail below

The exposures were proposed by me and consequently refined and finalised following discussion with a EuroSIDA working group of clinicians (J. Lundgren, O. Kirk), virologists (R. Paredes) and statisticians (A. Phillips and A. Cozzi-Lepri). It was decided to not study the influence of current VL in a time-updated manner, due to the difficulties in interpreting the results from such an analysis. This is because tests at higher VLs are more likely to amplify and, any routinely conducted tests which fail to amplify are not reported to the EuroSIDA resistance database. Therefore, resistance is by definition more likely to be detected at higher current VLs.

VL trajectories were estimated using univariable linear mixed models with a random intercept and slope for time, using all VL measurements that occurred between start of the VF episode and the last resistance test during a VF episode for each individual. VCY is a measure of cumulative VL exposure over time. Briefly, VCY is defined as the number of copies of VL circulating in plasma per year, integrated over time. Typically this is calculated using seroconversion as the origin time-point, when this is known (659). However, the same formula has been adapted to be used from other fixed timepoints (660). In this chapter, I calculated VCY from the start of the VF episode, which means that VCY represents the cumulative exposure to viral replication experienced from the start of VF up to the last documented resistance test during the VF. Using routinely collected data with VL levels only measured at specific time points, the integral is approximated using a time-weighted sum calculated using the trapezoidal rule (Equation 6.1).

Equation 6.1. Formula for the estimation of copy-years viraemia

$$\kappa_i(J_i) = \sum_{j=1}^{J_i} [t_i(j) - t_i(j-1)] \times [V_i(j) + V_i(j-1)]/2,$$

(659)

Where $K_i(J_i)$ represents copy years viremia in individual *i*, $V_i(j)$ and $V_i(j-1)$ are the viral load at respectively assessment *j* and assessment *j-1* for participant *i*, $t_i(j)$ and $t_i(j-1)$ are respectively the date of assessment *j* and *j-1* for individual *i.* If copy years viremia is calculated from infection, $t_i(0)$ is the date of HIV seroconversion and $V_i(0)$ is assumed 0. In practice, this is the equivalent of summing up for each individual the product of the mean of two consecutive VL measurements ($V_i(j)$ and $V_i(j-1)$). VCY for the first VL date and measurement in a series of measures is calculated by setting *t(j-1)* and *v(j-1)* to 0. The first VCY measure during each VF episode was therefore by definition zero.

There has been some discussion as to whether the VL measurements should be added together on a logarithmic or on a linear scale (660). Sempa et al conducted a literature review to study how the VCY has been estimated in different studies. Although a majority of studies using VCY add together measurements on a linear scale and consequently calculate the logarithm the sum, there are examples where this has not been done (660,661). Different approaches may lead to different estimates. Summing the measures on a linear scale gives greater weight to higher VL measurements, whereas summing under the log VL curve gives greater weight to repeated measures of intermediate values (660). Sempa et al compared the effect of VCY calculated both ways on mortality, and found that only VCY calculated by summing under the log VL curve was predictive of mortality. In the light of this, they recommend taking the 10^{th} logarithm of the VL measurements before applying the trapezoidal rule and that is the approach I chose to follow. I calculated the VCY for the entire follow-up time and used this as a constant, rather than time-updated, predictor in all models. Due to the nature of my study design, all individuals by definition reached the pre-specified study end-point (receiving a resistance test), and therefore, using the sum of the VCY per episode rather than a time-updated measure should not produce invalid results due to conditioning on future events.

The first three VL exposures were classified according to pre-specified VL thresholds broadly following prior WHO thresholds for defining VF: 51-500; 501-1,000; 1,001-5,000; 5,001-10,000; 10,001-100,000 and >100,000 copies/mL (72). It was decided a-priori to model these as categorical variables and to use the "5,001-10,000" category as the start of the VF episode category on the basis of the results from the literature review. VL trajectories and VCY were instead grouped using the quartiles of the distribution (Table 6.2). For the VL slope and VCY exposures, the lowest category (eg slopes less steep than +0.12 $\log_{10}$ copies/mL/year and VCY lower than 0.94 $\log_{10}$ copy × year/mL) was used as the baseline category. Quartiles and medians were determined per episode rather than per individual.

Table 6.2 VL exposure categories

Exposure (unit)	**Categories**
Start of the VF episode VL (copies /mL)	51-500; 501-1,000; 1,001-5,000; 5,001-10,000; 10,001-100,000 and >100,000
Average VL (copies /mL)	51-500; 501-1,000; 1,001-5,000; 5,001-10,000; 10,001-100,000 and >100,000
Peak VL (copies /mL)	51-500; 501-1,000; 1,001-5,000; 5,001-10,000; 10,001-100,000 and >100,000

VL slope (log_{10} copy /mL/year)	<Q1 (<0.12), Q1-M (0. 12-0.17) M-Q3 (0. 17-0.21), >Q3 (>0.21)
VCY (log_{10} copy × year/mL)	<Q1 (<0.94), Q1-M (0.94 -2.35) M-Q3 (2.35 -4.63), >Q3 (>4.63)

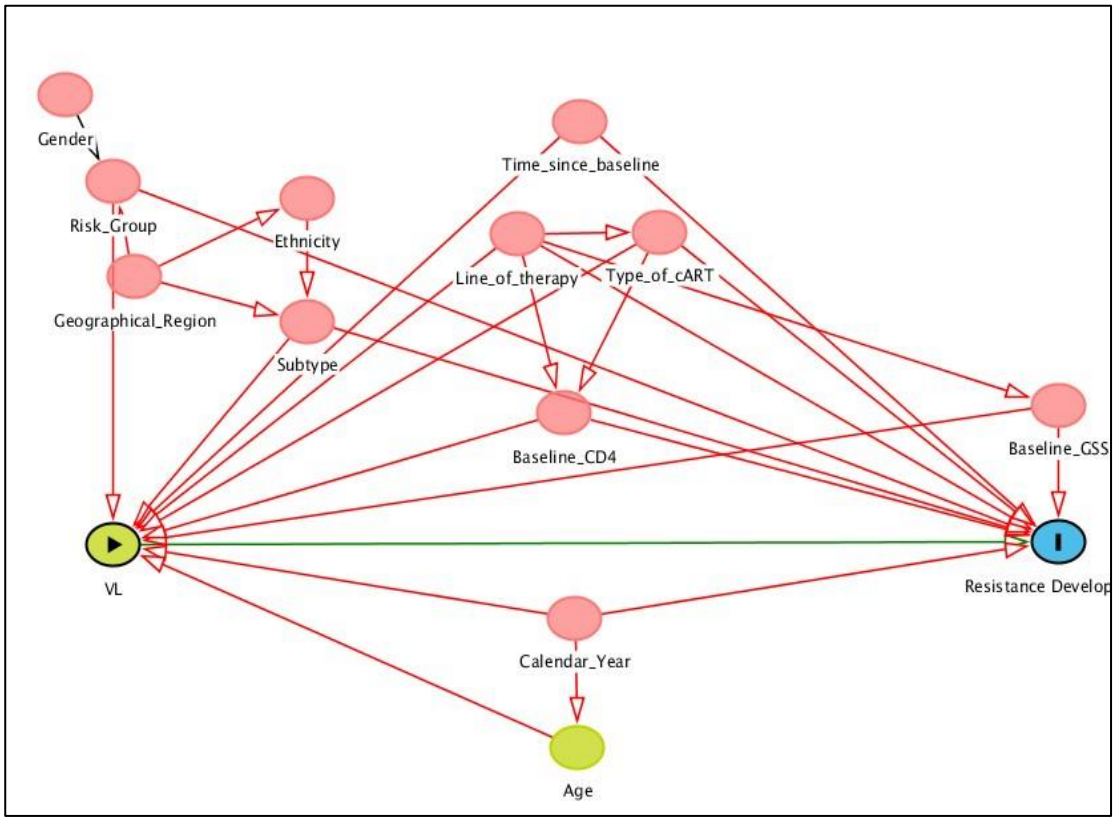
6.3.3. Resistance data

Resistance was defined using the IAS (2015) list (662). I did not consider as broad a definition of resistance as in Chapter 5, as the focus of this chapter lay on the risk of detection of any resistance rather than on individual mutations. Any resistance was defined as the detection of one or more IAS mutations, excluding minor PI mutations. A genotypic sensitivity score (GSS) was used as a secondary end-point, and the GSS was calculated using the ANRS (2015) interpretation rules (663). The level of resistance at the start of the VF episode was estimated in a cumulative manner using all resistance tests available before the start of the VF episode. For those experiencing the first VF from ART-naïve where no previous resistance tests were available, individuals were presumed to have no resistance mutations. If more than one resistance test was done after the start and during the same failure episode, both were used to determine the number of new mutations and the FU time censored at the date of the latest resistance test.

6.3.4. Covariate selection and categorisation

Besides viral load exposure, other factors were been considered as covariate as they were considered to be potential confounders of the association between viral load and the risk of accumulation of resistance. The final set of covariates has been selected on the basis of the results from the literature review and data availability in EuroSIDA. As the main interest was trying to assess causality, the selection of the final model was based on a directed acyclic graph (DAG) or causal diagram, shown below in Figure 6.4.

Figure 6.4. DAG for the effect of VL on resistance development



This DAG implies that the CD4 at the start of the VF episode CD4 (indicated as "Baseline_CD4"), line of therapy at the start of the episode, HIV subtype, mode of HIV transmission (indicated as "Risk_group"), calendar year of the episode start date, GSS of the failing regimen (indicated as "Baseline_GSS) and the duration of the episode (indicated as "Time_since_baseline") are independent predictors of VL and resistance development Consequently, these variables should be included in the multivariable model in order to estimate the total direct effect of VL on resistance development. The selected variables, together with their a priori categorisation, can be seen in Table 4.5.

Table 6.3 Categorisation of potential confounders included in the multivariable model

Variable	**Categories**	**Time-updated**
Mode of HIV transmission	MSM, PWID, Heterosexual, Other	N/A
HIV Subtype	B, Non-B	N/A
cART type	NNRTI-based, Unboosted PI-based, Boosted PI-based, Other three drug regimen, Mono/Dual	N/A
Calendar Year	96-97,98-99, 00-01, 02-03, 04-05, 06-07, 08-09, >10	No
CD4 count at the start of the failure episode	<200, 200-350, 350-500, >500	No

Duration of the episode	<4 months, 4-8 months, 8-16 months, >16 months	Yes
Line of therapy at the start of the episode[1]	1,2-3, 4-5, >5	N/A
GSS at the start of the episode	2 or lower, 3 or higher	No

1. Any change or gap in a regimen was counted as a new line of a regimen

The first three cART categories (NNRTI, unboosted PI and ritonavir boosted PI) were defined as those receiving exactly 3 drugs, of which 2 were NRTI and the remaining drug was either an NRTI, boosted PI or a ritonavir boosted PI. Ritonavir was not counted as a separate drug. Although CD4 is likely to affect both VL and possible resistance development in a time-updated manner, adjustment for this type of time-dependent confounding requires the use of more advanced statistical techniques, such as marginal structural models. This is because CD4 count is a time-dependent confounder affected by prior treatment as well as prior viral load values. As this lay outside the scope of this thesis it was decided to proceed with adjustments using start of the VF episode CD4 values only.

6.3.5. Statistical methods

Characteristics of the included participants at the start of the failure episode and during follow-up was summarised descriptively. The rate of resistance development was calculated as the number of new mutations to the failing regimen detected during consequent resistance tests divided by the time between start of the VF episode and the final resistance test (Person Years of Follow-Up, PYFU). Multivariable Poisson models with generalised estimating equations (GEE) were used to model the rate of resistance development. GEE were used to correct for the fact that episodes coming from the same participants cannot be assumed to be independent. Separate models were constructed for each VL exposure variable of interest.

6.3.6. Sensitivity analyses

The analysis was repeated with the following variations:

1. Counting only the mutations that were classed as conferring resistance to the specific regimen an individual was maintained when constructing the outcome.
2. Using mutations from each main class of drug resistance (NRTI, NNRTI and PI) as the outcome.

6.4. Results

6.4.1. Characteristics of the study population

Overall, 464 individuals contributing a total of 549 episodes were included. 159 (34%) individuals experienced VF while receiving their first ART regimen started from ART-naive. 40 (25%) of these nonetheless had a previous resistance test which allowed for an estimation of their start of the VF episode resistance patterns, for the others I assumed that no transmitted resistance was present. The median duration of the episodes was 8 (Inter Quartile Range [QR]=4-16) months, with a median of 1 (IQR=1-1) resistance tests and 3 (IQR=2-6) VL measurements taken during the episode. The characteristics of individuals at start date of the failure episode can be seen in Table 6.4.

Table 6.4. Characteristics of the study population at the start of VF

Per individual (N=464)		**Total N (%)**
Gender	Male	374 (80.6)
	Female	90 (19.4)
Age, years	Years, Median(IQR)	40 (34, 47)
Mode of HIV transmission	MSM	228 (52.1)
	PWID	76 (17.4)
	Heterosexual	122 (27.9)
	Other	12 (2.7)
Ethnicity	White	387 (84.3)
	Other	72 (15.7)
Subtype B	B	354 (84.7)
	Non-B	64 (15.3)
VL	cp/ml, Median(IQR)	4150 (717, 32450)
CD4	cells/mm^3, Median(IQR)	259 (158, 407)
Line of regimen	Number, Median (IQR)	5 (1, 9)
Type of cART regimen	NNRTI-based	52 (11.2)
	Unboosted PI-based	93 (20.0)
	Boosted PI-based	82 (17.7)
	Other	126 (27.2)
	Mono/Dual	111 (23.9)
Number of drugs in the cart regimen	Number, Median (IQR)	3 (3, 3)
Calendar year	Month/Year, Median (IQR)	02/00 (10/97-10/02)
Per episode (N=549)		
Duration of episodes	Months, Median (IQR)	8 (4, 16)
Resistance tests	Number, Median (IQR)	1 (1, 1)
VL measurements	Number, Median (IQR)	3 (2, 6)

The majority (80.6%) of individuals were male, and the median age of the study population was 40 (IQR-34-47) years. The majority acquired HIV through sex with another man (52.1%), and the vast majority were of white ethnicity (84.3%) and were infected with a subtype B virus

(84.7%). Most individuals experienced VF episodes in early calendar years, with the median date of the start of the VF episode date February 2000 (IQR=October 1997-October 2002). The median CD4 count at start of the VF episode was 259 (IQR=158-407) cells/mm^3, and this did not differ markedly compared to the median CD4 count at the end of episodes (257, IQR=140-407 cells/mm^3).

The most common type of cART received at start of the VF episode was "Other" regimens (27.2%), which included all regimens composed of more than 3 drugs. However, receiving mono/dual therapy was also relatively common (23.9%), as was receiving cART with an unboosted PI (20.0%). The median number of drugs received was 3 (IQR=3-3). 14 episodes (2.6%) occurred while the individual was receiving monotherapy. Regimens taken by 1% (N=5) or more of the study population can be seen in Table 6.5 below.

Table 6.5. Most common regimens used at start of the VF episode

Regimen	N	%
Zidovudine /Lamivudine	27	5.82
Zidovudine/ Lamivudine /Indinavir	26	5.6
Lamivudine /Stavudine/ Indinavir	18	3.88
Zidovudine /Didanosine	17	3.66
Lamivudine / Stavudine / Saquinavir /Ritonavir	14	3.02
Zidovudine / Lamivudine /Nevirapine	12	2.59
Lamivudine / Stavudine /Nelfinavir	11	2.37
Zidovudine /Zalcitabine	11	2.37
Lamivudine / Stavudine	9	1.94
Zidovudine / Lamivudine / Nelfinavir	9	1.94
Zidovudine / Lamivudine /Efavirenz	7	1.51
Didanosine / Stavudine	6	1.29
Didanosine / Stavudine / Nevirapine	6	1.29
Lamivudine / Stavudine / Nevirapine	6	1.29
Lamivudine / Stavudine / Nevirapine / Nelfinavir	6	1.29
Zidovudine / Lamivudine /Abacavir	6	1.29
Zidovudine / Lamivudine / Saquinavir	6	1.29
Lamivudine / Stavudine / Ritonavir	5	1.08
Tenofovir/ Ritonavir /Atazanvir/Emtricitabine	5	1.08
Zidovudine	5	1.08
Zidovudine / Zalcitabine /Saquinavir	5	1.08

The most common regimen was dual therapy with Zidovudine and Lamivudine, followed by triple therapy of Zidovudine, Lamivudine and Indinavir. Stavudine (D4T) featured relatively frequently in the regimens, and only 2 individuals were on current WHO recommended first line therapy (TDF+FTC+EFV), which prevented me from investigating resistance accumulation among these individuals alone. The treatment patterns are likely to reflect the fact that most

of the start dates of the episodes occurred relatively soon after or before cART was made widely available.

A breakdown of the frequency distribution of the episodes according to values of the VL exposures considered can be seen in Table 6.6. Most individuals had both start of the VF episode VL (29.7%), average VL (39.7%) and peak VL (36.0%) values of between 10,001 and 100,000 copies/mL. Very few individuals had peak VL values below 1,001 copies/ml (N=30). The vast majority of individuals had positive values of the VL slope (indicating that the VL was increasing during the episode), with only seven individuals (1.5%) estimated to have decreasing VL values between the start and end of their VF episode. Because so few individuals had a decreasing slope, it was not possible to model these in a separate category.

Table 6.6. Values of the VL exposure variables at start of the VF episode

Exposure Variable	Category	N	%
Start of the VF episode VL (copies /mL)	51-500	86	18.5
	501-1,000	45	9.7
	1,001-5,000	116	25.0
	5,001-10,000	30	6.5
	10,001-100,000	138	29.7
	>100,000	49	10.6
Average VL (copies /mL)	51-500	29	6.3
	501-1,000	34	7.3
	1,001-5,000	118	25.4
	5,001-10,000	55	11.9
	10,001-100,000	167	36.0
	>100,000	61	13.2
Peak VL (copies /mL)	51-500	12	2.6
	501-1,000	18	3.8
	1,001-5,000	87	18.8
	5,001-10,000	48	10.3
	10,001-100,000	184	39.7
	>100,000	115	24.8
VL slope (log_{10} cp/mL/year)	<Q1 (<0.12)	111	23.9
	Q1-M (0. 12-<0.17)	110	23.7
	M-Q3 (0. 17-<0.21)	121	26.1
	>Q3 (>0.21)	122	26.3
VCY (log_{10} cp * year/mL)	<Q1 (<0.94)	118	25.4
	Q1-M (0.94 -<2.35)	109	23.5
	M-Q3 (2.35 -<4.63)	113	24.4
	>Q3 (>4.63)	124	26.7

6.4.2. Prevalence of resistance

The prevalence of resistance to any drug at the start of episodes was 71%, and this had increased to 92% by the end of episodes (Figure 6.5). At least one new resistance mutation accumulated in 57% of all included episodes. On average, NRTI resistance was most commonly

detected both at the start (67%) and at the end of episodes (88%). The prevalence of NNRTI and PI resistance was nearly equal at the start of episodes (46% respectively), but NNRTI resistance was slightly more common than PI resistance at the end of episodes (60% v 58%). The prevalence of individual NRTI, NNRTI and PI mutations can be seen in Figure 6.6-Figure 6.8. The most common NRTI resistance mutation was M184V, K103N the most common NNRTI mutation and L90M the most common PI mutation.

Figure 6.5. Prevalence of any resistance and resistance respectively to NRTI, NNRTI and PI at the beginning and end of a VF episode

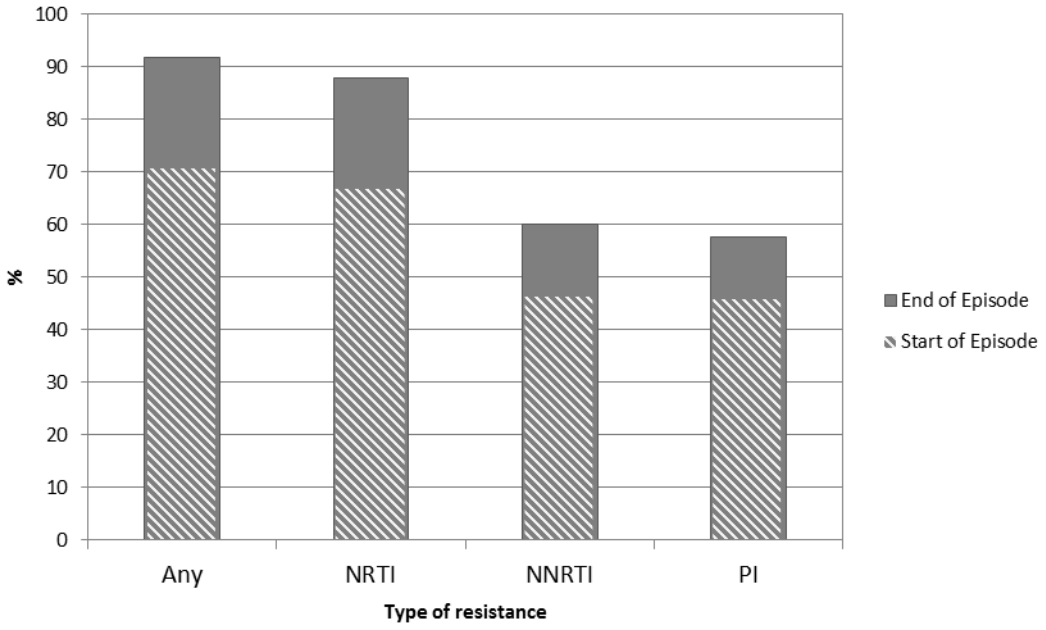


Figure 6.6. Prevalence of NRTI resistance mutations at the beginning and end of a VF episode

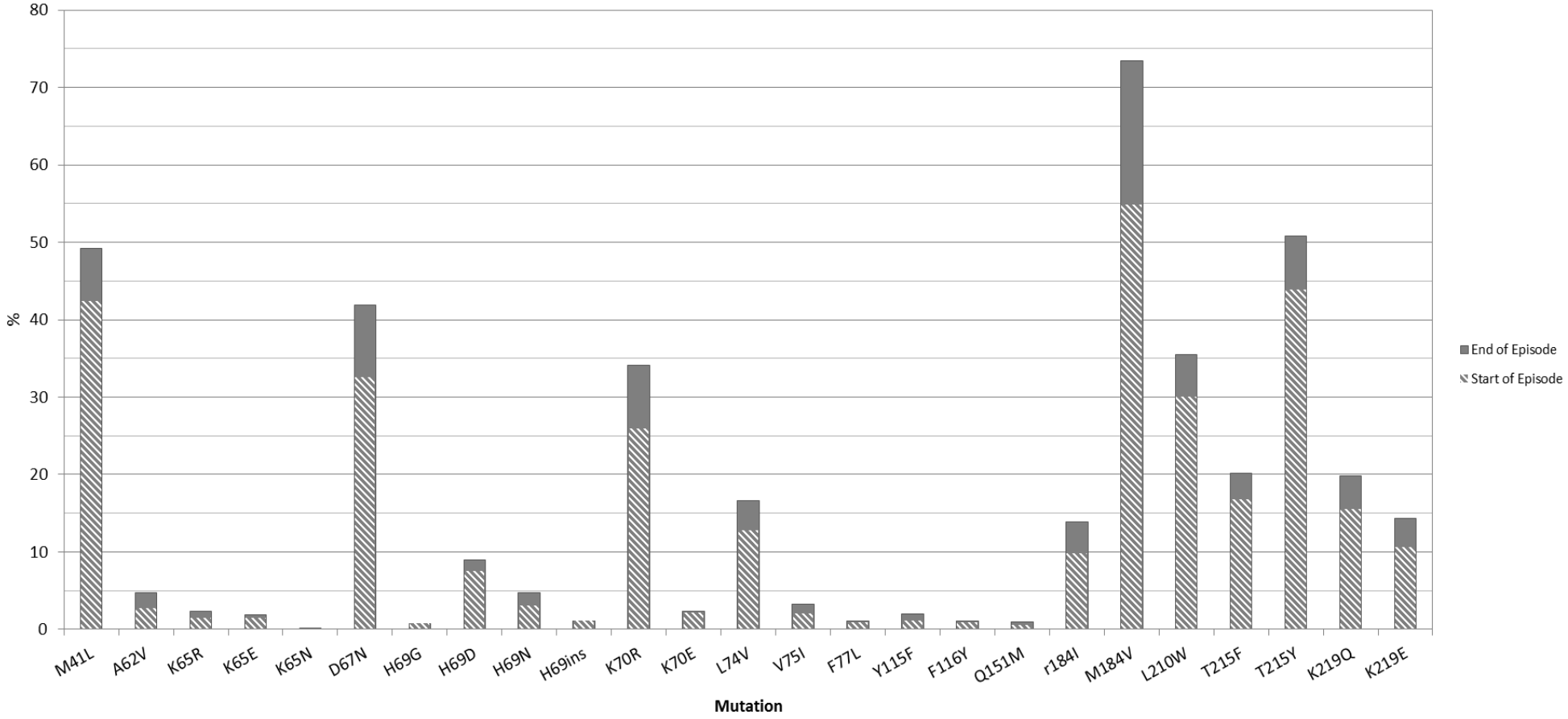


Figure 6.7. Prevalence of NNRTI resistance mutations at the beginning and end of a VF episode

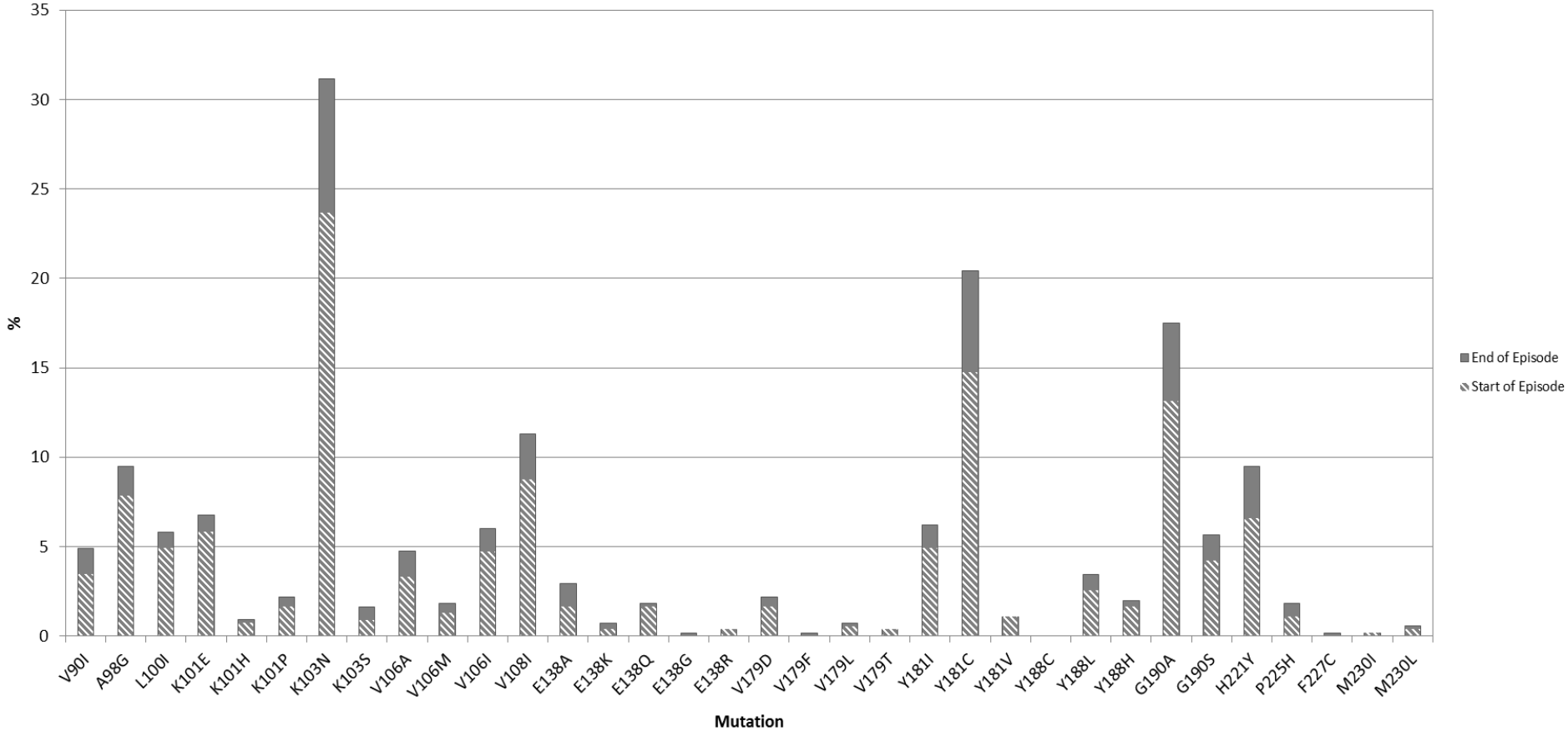
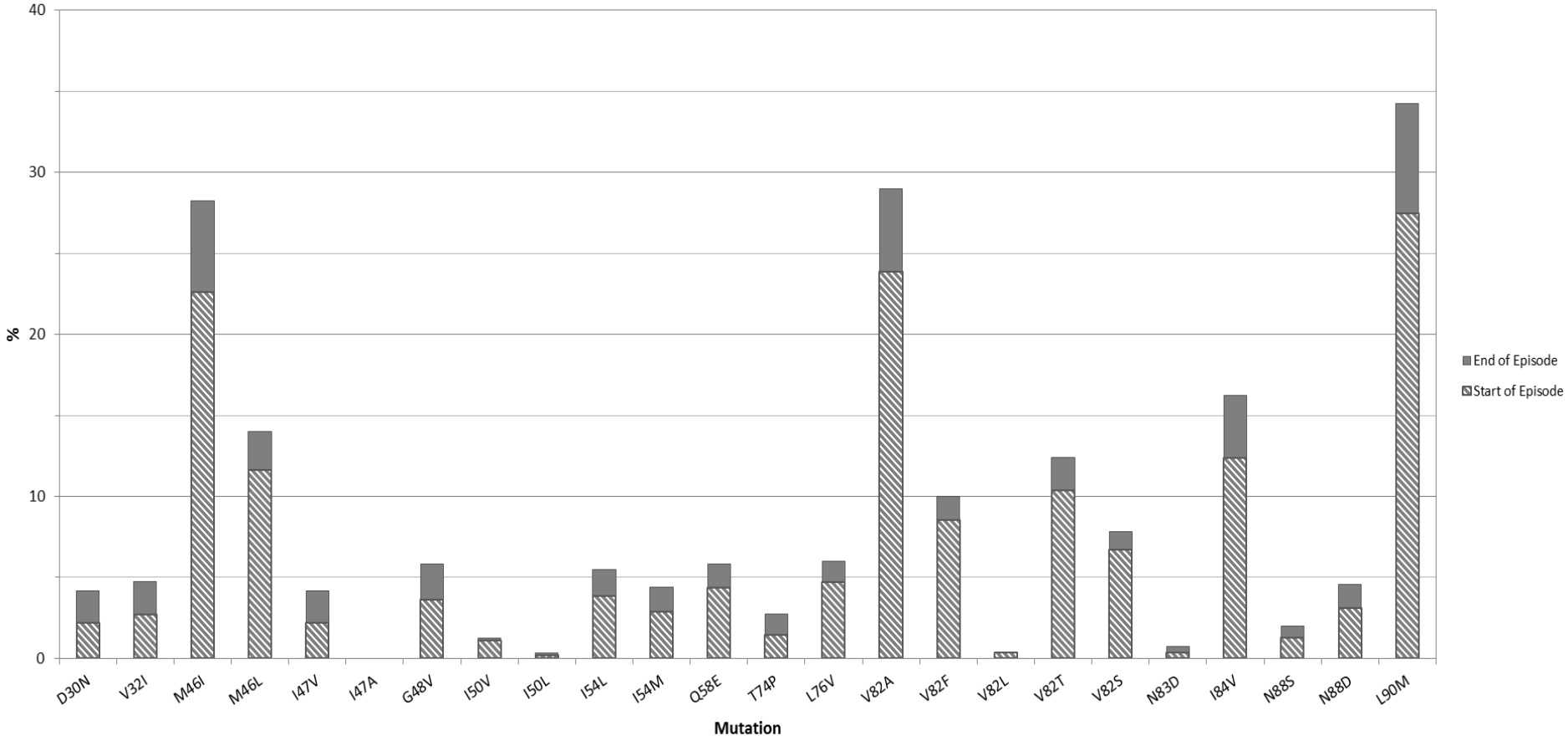


Figure 6.8. Prevalence of PI resistance mutations at the beginning and end of a VF episode



6.4.3. Crude rates of resistance accumulation

Overall, 932 new IAS mutations accumulated during 615 person years of follow-up, leading to an overall crude rate of resistance development of 1.51 (95%CI=1.37-1.68) new mutations per year. This is the equivalent to one new mutation accumulating per every 6.7 months of an individual being kept on a failing regimen. The equivalent rate of GSS reduction was 0.49 (95%CI=0.43-0.56), or the equivalent of one virologically active drug (which formed part of the failing regimen) lost as a result of every two years of someone being maintained on the same failing treatment regimen.

The rate of accumulation of any, class, and individual mutations where at least one mutation accumulated can be seen in Table 6.7 below.

Table 6.7. Rates of accumulation of any, class and individual mutations over the

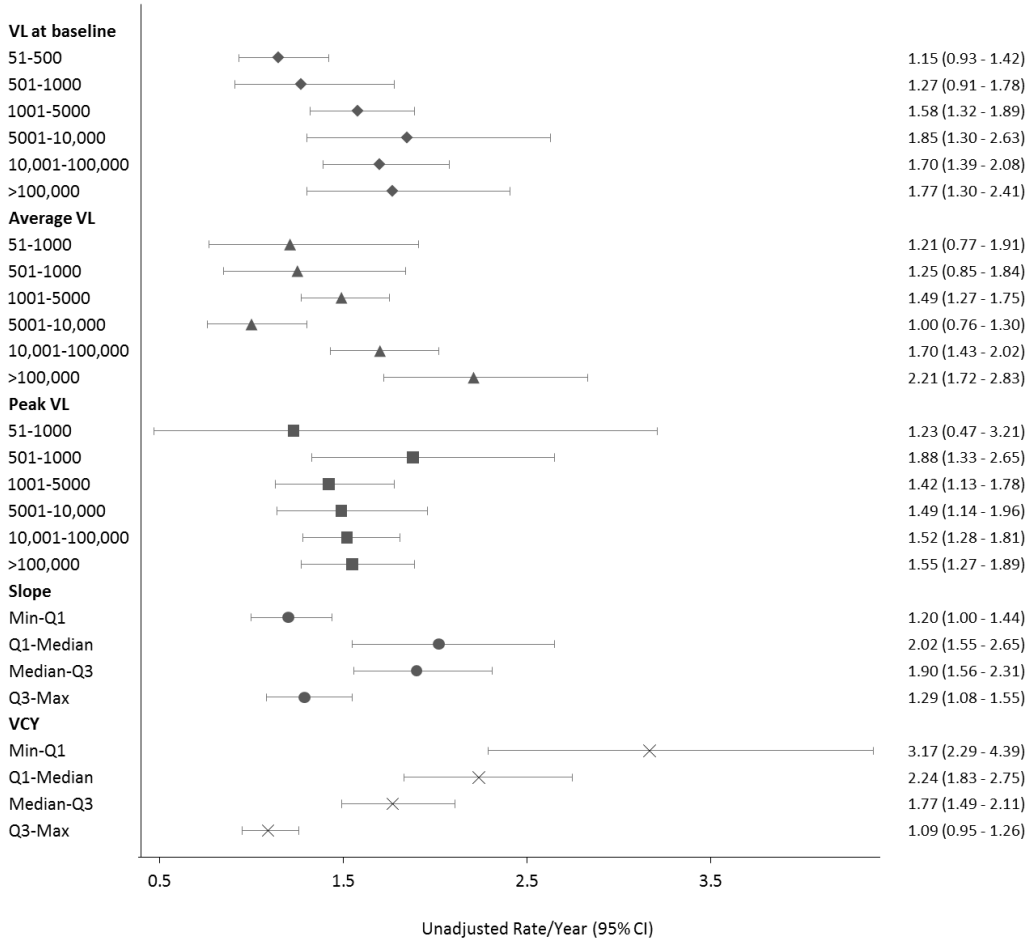
	N	Rate/year (95%CI)
	932	**1.52 (1.37 - 1.68)**
NRTI	462	0.75 (0.67-0.85)
NNRTI	223	0.36 (0.30-0.44)
PI	247	0.40 (0.34-0.48)
	N	**Rate/10 years (95%CI)**
RT mutations		
M41L	37	0.6 (0.45-0.81)
A62V	11	0.18 (0.1-0.32)
K65R	5	0.08 (0.03-0.2)
K65E	2	0.03 (0.01-0.13)
D67N	51	0.83 (0.64-1.07)
H69D	8	0.13 (0.07-0.26)
H69N	9	0.15 (0.08-0.28)
K70E	1	0.02 (0-0.12)
K70R	45	0.73 (0.56-0.96)
I74V	21	0.34 (0.23-0.52)
V75I	7	0.11 (0.05-0.24)
F77L	1	0.02 (0-0.12)
Y115F	5	0.08 (0.03-0.19)
F116Y	1	0.02 (0-0.11)
Q151M	2	0.03 (0.01-0.13)
M184I	22	0.36 (0.24-0.54)
M184V	102	1.66 (1.38-2)
L210W	30	0.49 (0.35-0.68)
T215F	19	0.31 (0.2-0.48)
T215Y	38	0.62 (0.46-0.83)
K219Q	24	0.39 (0.27-0.57)
K219E	21	0.34 (0.23-0.52)
V90I	8	0.13 (0.06-0.26)
A98G	9	0.15 (0.08-0.28)
L100I	5	0.08 (0.03-0.19)
K101E	5	0.08 (0.03-0.19)
K101H	1	0.02 (0-0.12)

K101P	3	0.05 (0.02-0.15)
K103N	41	0.67 (0.49-0.9)
K103S	4	0.07 (0.02-0.17)
V106A	8	0.13 (0.07-0.26)
V106M	3	0.05 (0.02-0.15)
V106I	7	0.11 (0.05-0.24)
V108I	14	0.23 (0.14-0.38)
E138A	7	0.11 (0.05-0.24)
E138K	2	0.03 (0.01-0.13)
E138Q	1	0.02 (0-0.12)
E138G	1	0.02 (0-0.11)
V179D	3	0.05 (0.02-0.15)
V179F	1	0.02 (0-0.12)
V179L	1	0.02 (0-0.12)
Y181I	7	0.11 (0.06-0.24)
Y181C	31	0.5 (0.36-0.71)
Y188L	5	0.08 (0.03-0.2)
Y188H	2	0.03 (0.01-0.13)
G190A	24	0.39 (0.26-0.58)
G190S	8	0.13 (0.07-0.26)
H221Y	16	0.26 (0.16-0.42)
P225H	4	0.07 (0.02-0.17)
F227C	1	0.02 (0-0.12)
M230L	1	0.02 (0-0.12)
PI mutations		
D30N	11	0.18 (0.1-0.32)
V32I	11	0.18 (0.1-0.32)
M46I	31	0.5 (0.36-0.71)
M46L	13	0.21 (0.12-0.36)
I47V	11	0.18 (0.1-0.32)
G48V	12	0.2 (0.11-0.34)
I50V	1	0.02 (0-0.12)
I50L	1	0.02 (0-0.12)
I54L	9	0.15 (0.08-0.28)
I54M	8	0.13 (0.07-0.26)
Q58E	8	0.13 (0.07-0.26)
T74P	7	0.11 (0.05-0.24)
L76V	7	0.11 (0.05-0.24)
V82A	28	0.46 (0.32-0.66)
V82F	8	0.13 (0.07-0.25)
V82T	11	0.18 (0.1-0.32)
V82S	6	0.1 (0.05-0.21)
N83D	2	0.03 (0.01-0.13)
I84V	21	0.34 (0.22-0.52)
N88S	4	0.07 (0.02-0.17)
L90M	37	0.6 (0.44-0.82)

NRTI mutations accumulated at a rate of 0.75 (95%CI=0.67-0.85) new mutations/year, NNRTI mutations at 0.36 (95%CI=0.30-0.44) new mutations/year and major PI mutations at a rate of 0.40 (95%CI=0.34=0.48) new mutations/year. The individual mutation that accumulated the fastest was M184V at 0.17 (95%CI-0.14-0.20)/year.

The rate of resistance accumulation according to the various measures VL exposure can be seen in Figure 6.9.

Figure 6.9. Crude rates (95% CI)/year of resistance development according to VL exposure



The VL level at the start of a VF episode was borderline associated with resistance development (p=0.07). The rates were higher with higher levels of VL up until VL values between 5,001-10,000 copies/mL. In categories above this the rate of resistance development was relatively stable. There was also an association between average VL levels and resistance accumulation (p=0.005), with resistance accumulation being considerably higher at VLs in the 10,001-100,0000 category and at VLs above 100,000. In contrast to the association with start of the VF episode VL, the 5,001-10,000 category had the lowest rates of resistance development.

However, the rate of accumulation was not associated with the level of peak VL (p=0.87). The rate of resistance accumulation also varied according to both the slope of VL change between the start and the end of episodes (p=0.009) and according to VCY (p<0.001). Accumulation was highest at low to intermediate VL slope values (Q1-Median), and was considerably lower in the lowest (Min-Q1) and highest (Q3-Max) categories. In contrast, accumulation of resistance appeared to decrease in a linear fashion according to increasing VCY.

Rates of resistance accumulation also varied according to a number of the selected covariates (Table 6.8). Specifically, there was evidence that the rate of accumulation was lower among individuals receiving boosted PI therapy compared to other drug classes (p=0.003), individuals with CD4 counts >500 cells/mm^3 compared to those with lower CD4 counts (p=0.006) and those infected with a subtype non-B virus compared to a B virus (p=0.009). Resistance accumulation was higher during episodes of long duration (p<0.001) and among individuals with lower GSS at the start of the VF episode (p<0.001).

Table 6.8. Rates (95%CI) according to values of the selected covariates

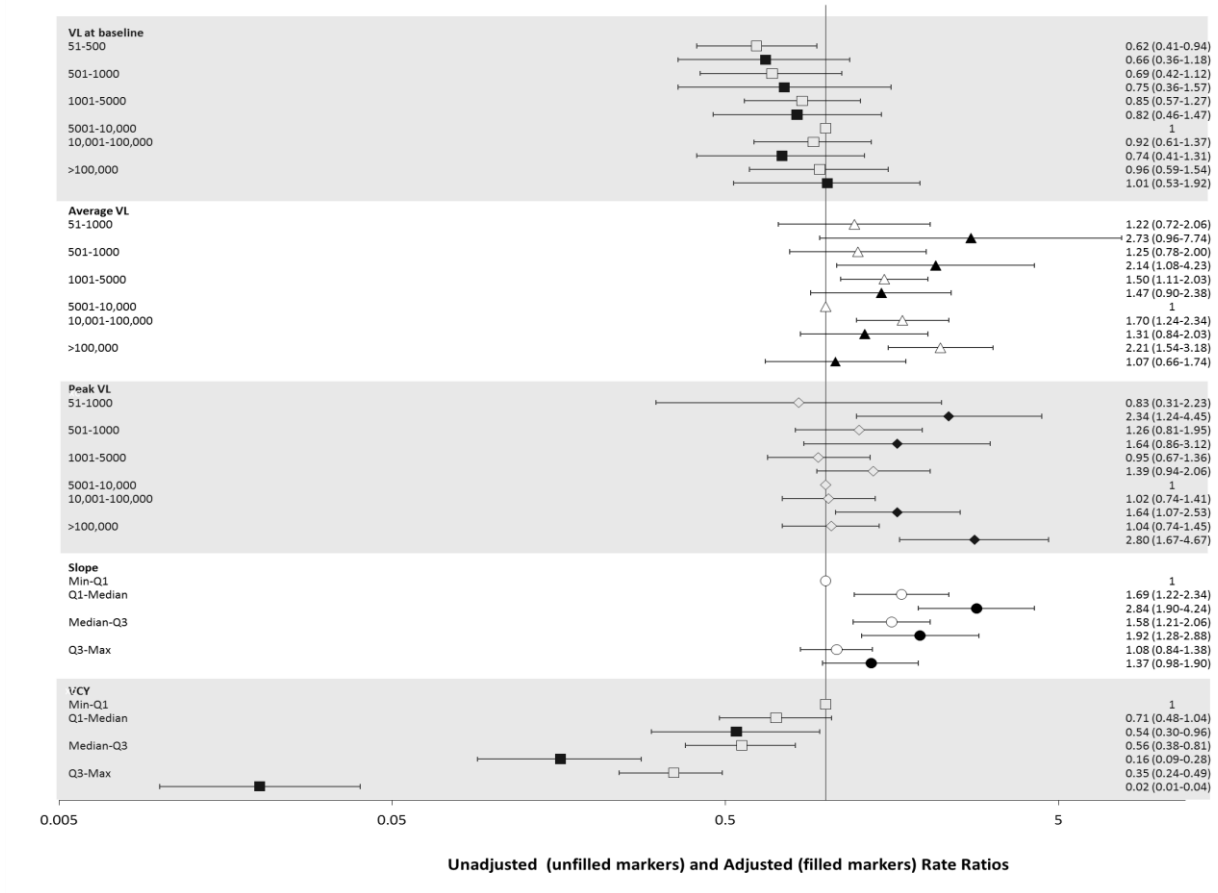
		N	PYFU	Crude Rate (95%CI)/year	P
Mode of HIV transmission	MSM	523	330	1.58 (1.37 - 1.84)	0.54
	PWID	130	86	1.51 (1.18 - 1.92)	
	Heterosexual	203	155	1.31 (1.07 - 1.61)	
	Other	22	15	1.46 (0.81 - 2.63)	
cART Type	NNRTI	151	75	2.03 (1.60 - 2.57)	0.003
	Unboosted	170	91	1.88 (1.55 - 2.28)	
	Boosted PI	120	111	1.08 (0.80 - 1.45)	
	Mono/Dual	246	160	1.54 (1.30 - 1.83)	
	Other	245	179	1.37 (1.09 - 1.72)	
ART-Naive	Yes	581	382	1.52 (1.35 - 1.72)	0.96
	No	351	232	1.51 (1.25 - 1.82)	
Calendar Year of VF	96-97	85	71	1.19 (0.83 - 1.72)	0.18
	98-99	250	142	1.76 (1.40 - 2.21)	
	00-01	177	145	1.22 (0.93 - 1.60)	
	02-03	167	115	1.45 (1.07 - 1.96)	
	04-05	162	87	1.85 (1.38 - 2.49)	
	06-07	69	34	2.00 (1.24 - 3.23)	
	08-09	9	11	0.84 (0.31 - 2.28)	
	>10	13	8	1.59 (0.60 - 4.24)	
CD4 count at start of the VF episode	<200	318	157	2.03 (1.71 - 2.40)	0.006
	200-350	257	188	1.37 (1.09 - 1.71)	
	350-500	196	134	1.46 (1.10 - 1.94)	
	>500	161	135	1.19 (0.96 - 1.48)	
Line of Therapy	1	313	217	1.44 (1.17 - 1.77)	0.55
	2-3	106	55	1.91 (1.39 - 2.64)	
	4-5	118	78	1.44 (1.17 - 1.77)	
	>5	395	264	1.91 (1.39 - 2.64)	

Time since start of the VF episode	< 4 months	124	167	0.74 (0.52 - 1.05)	<0.00111
	4-8 months	162	120	1.35 (1.02 - 1.77)	
	8-16 months	237	143	1.66 (1.31 - 2.10)	
	>16 months	409	184	2.23 (1.83 - 2.71)	
Subtype	B	90	82	1.70 (1.52 - 1.90)	0.009
	Not-B	763	449	1.10 (0.81 - 1.49)	
GSS at start of the VF episode	2 or lower	911	497	1.83 (1.62 - 2.07)	<.0001
	3 or higher	21	117	0.18 (0.08 - 0.38)	

6.4.4. Univariable and multivariable models of resistance accumulation

After adjustment for DAG-identified potential confounders, average VL (p=0.009), VL slope (p<0.001) and VCY (p<0.001) remained associated with the risk of resistance development in a multivariable Poisson regression model (Figure 6.10). For the VL at the start of VF episode, although adjustments did not shift its pattern of association with resistance development markedly, the p-value grew considerably larger (p=0.58). For average VL, there was a more marked modification of the relationship with the risk of resistance accumulation from that observed in univariable analyses, although the lowest rate of resistance accumulation nonetheless occurred at intermediate values of average VL (5,001-10,000; *reference category,* Figure 6.10). In contrast, the multivariable models indicated that resistance accumulation was still higher at low-intermediate values of VL slope (0.12-<0.17 $\log_{10}$ copies/mL/year, 2.84, 95%CI=1.90-4.24), and lower at both the highest (0.17-12 $\log_{10}$ copies/mL/year, 1.37, 95%CI=0.98-1.90) and the lowest (<12 $\log_{10}$ copies/mL/year, *reference*) quartiles of estimated VL slopes. The sharp decline of resistance accumulation with increasing VCY was confirmed by multivariable modelling, with a rate ratio of only 0.02 (95%CI=0.01-0.04) in the highest quartile of VCY compared to the lowest.

Figure 6.10. Unadjusted and adjusted rate ratios for the development of resistance according to VL exposure



6.4.5. Sensitivity Analyses

I re-ran the analyses counting only mutations associated with reduced susceptibility to the exact drugs included in the regimen an individual was maintained on during the VF episode for the construction of the outcome variable. The results can be seen in Table 6.9-Table 6.10 below. Briefly, the overall rate of mutation accumulation was considerably lower when only taking mutations specific to the failing regimen into account, at 0.63 new mutations (95%CI=0.55-0.72) per year. However, the shape of the relationship between VL exposures and resistance accumulation remained the same, with an inversely U-shaped relationship between the VL slope and resistance development and a linearly decreasing relationship between increasing VCY and resistance development after adjustment. Although average VL was also associated with resistance accumulation, this relationship did not follow a clear shape.

Table 6.9. Rates of development of mutations specific to a given regimen according to VL

		N	FU	Crude Rate	P-value
		387	615	0.63 (0.55 - 0.72)	
Start of the VF episode VL (copies	51-500	57	138	0.41 (0.31 - 0.56)	0.003
	501-1,000	29	68	0.42 (0.28 - 0.65)	
	1,000-5,000	98	150	0.65 (0.51 - 0.85)	
	5,001-10,000	40	46	0.86 (0.56 - 1.32)	
	10,001-100,000	111	163	0.68 (0.53 - 0.88)	
	>100,000	52	49	1.06 (0.76 - 1.48)	
Average VL (copies /mL)	51-500	11	28	0.39 (0.20 - 0.77)	0.002
	501-1,000	24	47	0.52 (0.37 - 0.72)	
	1,000-5,000	91	161	0.57 (0.45 - 0.72)	
	5,001-10,000	38	100	0.38 (0.25 - 0.57)	
	10,001-100,000	159	227	0.70 (0.56 - 0.88)	
	>100,000	64	52	1.24 (0.92 - 1.67)	
Peak VL (copies /mL)	51-500	6	9	0.67 (0.25 - 1.80)	0.90
	501-1,000	12	18	0.66 (0.40 - 1.11)	
	1,000-5,000	61	99	0.62 (0.48 - 0.79)	
	5,001-10,000	38	66	0.58 (0.41 - 0.83)	
	10,001-100,000	158	267	0.59 (0.47 - 0.75)	
	>100,000	112	156	0.72 (0.56 - 0.92)	
VL slope (log_{10} cp /mL/year)	<Q1 (<0.12)	103	191	0.54 (0.42 - 0.69)	0.071
	Q1-M (0. 12-<0.17)	93	111	0.84 (0.61 - 1.16)	
	M-Q3 (0. 17-<0.21)	90	122	0.74 (0.56 - 0.97)	
	>Q3 (>0.21)	101	191	0.53 (0.42 - 0.67)	
VCY (log_{10} cp * year/mL)	<Q1 (<0.94)	59	37	1.58 (1.13 - 2.22)	<0.0001
	Q1-M (0.94 -<2.35)	83	76	1.09 (0.86 - 1.40)	
	M-Q3 (2.35 -<4.63)	106	141	0.75 (0.60 - 0.94)	
	>Q3 (>4.63)	139	360	0.39 (0.31 - 0.48)	

Table 6.10. Unadjusted and Adjusted Rate Ratios of the development of specific resistance mutations according to VL exposures

		Unadjusted RR	P	GP	Adjusted RR	P	GP[1]
Start of the VF episode VL (copies /mL)	51-500	0.48 (0.28 - 0.81)	0.006	0.003	0.72 (0.38 - 1.38)	0.327	0.316
	501-1,000	0.49 (0.27 - 0.90)	0.022		0.58 (0.26 - 1.28)	0.174	
	1,000-5,000	0.76 (0.46 - 1.25)	0.282		0.90 (0.49 - 1.66)	0.742	
	5,001-10,000	*1.00*			*1.00*		
	10,001-100,000	0.79 (0.48 - 1.29)	0.349		0.72 (0.39 - 1.33)	0.293	
	>100,000	1.23 (0.71 - 2.14)	0.464		1.25 (0.64 - 2.44)	0.506	
Average VL (copies /mL)	51-500	1.04 (0.47 - 2.28)	0.928	0.002	3.98 (1.14 - 13.87)	0.030	0.027
	501-1,000	1.36 (0.80 - 2.32)	0.256		2.49 (1.09 - 5.71)	0.031	
	1,000-5,000	1.49 (0.93 - 2.39)	0.094		1.79 (1.01 - 3.18)	0.047	
	5,001-10,000	*1.00*			*1.00*		
	10,001-100,000	1.85 (1.15 - 2.97)	0.011		1.47 (0.88 - 2.45)	0.140	
	>100,000	3.27 (1.96 - 5.47)	<.001		1.19 (0.68 - 2.10)	0.542	
Peak VL (copies /mL)	51-500	1.16 (0.41 - 3.31)	0.779	0.899	2.42 (1.10 - 5.29)	0.028	0.144
	501-1,000	1.15 (0.61 - 2.15)	0.672		1.54 (0.71 - 3.35)	0.275	
	1,000-5,000	1.06 (0.69 - 1.64)	0.787		1.44 (0.85 - 2.42)	0.173	
	5,001-10,000	*1.00*			*1.00*		
	10,001-100,000	1.02 (0.67 - 1.57)	0.921		1.72 (0.98 - 3.02)	0.061	
	>100,000	1.24 (0.81 - 1.91)	0.327		3.24 (1.71 - 6.13)	<.001	
VL slope (log_{10} cp/mL/year)	<Q1	*1.00*			*1.00*		
	Q1-M	1.56 (1.04 - 2.34)	0.032	0.071	2.34 (1.50 - 3.65)	<.001	0.005
	M-Q3	1.36 (0.94 - 1.98)	0.102		1.48 (0.88 - 2.50)	0.141	
	>Q3	0.98 (0.70 - 1.37)	0.911		1.25 (0.82 - 1.90)	0.299	
	<Q1	*1.00*			*1.00*		

VCY (log_{10} cp * year/mL)	Q1-M	0.69 (0.45 - 1.06)	0.092	<.001	0.57 (0.30 - 1.08)	0.085	<.001
	M-Q3	0.47 (0.32 - 0.71)	<.001		0.14 (0.08 - 0.25)	<.001	
	>Q3	0.24 (0.16 - 0.36)	<.001		0.02 (0.01 - 0.04)	<.001	

1. GP=Global P

I also evaluated the using class-specific resistance accumulation as the outcome (Table 6.11-Table 6.12). There were some differences compared to the primary analysis: notably, average VL was not associated with the accumulation of NRTI or NNRTI resistance, and only weakly associated with the accumulation of PI resistance. However, the absolute values of the rate ratios were reasonably similar across both the main and sensitivity analysis. The associations between the VL slope and VCY and accumulation of resistance also remained similar when considering NRTI, NNRTI and PI resistance separately.

Table 6.11. Rates of resistance accumulation of class-specific mutations according to VL

		NRTI		NNRTI		PI	
		Crude Rate (95%CI)	**P-value**	**Crude Rate (95%CI)**	**P-value**	**Crude Rate (95%CI)**	**P-value**
Start of the VF episode VL (copies /mL)	51-500	0.65 (0.50 - 0.83)	0.69	0.17 (0.11 - 0.29)	0.02	0.33 (0.22 - 0.49)	0.06
	501-1,000	0.73 (0.51 - 1.05)		0.37 (0.22 - 0.61)		0.18 (0.09 - 0.36)	
	1,000-5,000	0.82 (0.66 - 1.02)		0.39 (0.28 - 0.56)		0.37 (0.25 - 0.54)	
	5,001-10,000	0.75 (0.50 - 1.13)		0.54 (0.30 - 0.96)		0.56 (0.31 - 1.00)	
	10,001-100,000	0.82 (0.63 - 1.07)		0.39 (0.27 - 0.55)		0.49 (0.37 - 0.65)	
	>100,000	0.63 (0.39 - 1.03)		0.55 (0.33 - 0.93)		0.59 (0.34 - 1.02)	
Average VL (copies /mL)	51-500	0.79 (0.44 - 1.40)	0.61	0.11 (0.02 - 0.49)	0.01	0.32 (0.16 - 0.64)	0.035
	501-1,000	0.84 (0.54 - 1.30)		0.26 (0.14 - 0.47)		0.15 (0.06 - 0.37)	
	1,000-5,000	0.76 (0.63 - 0.92)		0.36 (0.26 - 0.49)		0.37 (0.26 - 0.53)	
	5,001-10,000	0.59 (0.44 - 0.78)		0.21 (0.11 - 0.39)		0.20 (0.11 - 0.35)	
	10,001-100,000	0.81 (0.65 - 1.00)		0.40 (0.30 - 0.54)		0.49 (0.37 - 0.65)	
	>100,000	0.72 (0.45 - 1.13)		0.74 (0.51 - 1.07)		0.75 (0.51 - 1.11)	
Peak VL (copies /mL)	51-500	0.56 (0.20 - 1.54)	0.77	0.34 (0.07 - 1.55)	0.84	0.34 (0.12 - 0.98)	0.65
	501-1,000	1.05 (0.62 - 1.79)		0.28 (0.11 - 0.67)		0.55 (0.29 - 1.06)	
	1,000-5,000	0.83 (0.63 - 1.09)		0.31 (0.20 - 0.49)		0.28 (0.17 - 0.47)	
	5,001-10,000	0.69 (0.49 - 0.97)		0.38 (0.24 - 0.60)		0.43 (0.25 - 0.72)	
	10,001-100,000	0.76 (0.63 - 0.92)		0.34 (0.25 - 0.46)		0.42 (0.32 - 0.56)	
	>100,000	0.69 (0.53 - 0.91)		0.44 (0.31 - 0.61)		0.42 (0.30 - 0.60)	
VL slope (log_{10} cp/mL/year)	<Q1 (<0.12)	0.56 (0.44 - 0.71)	<0.001	0.29 (0.20 - 0.42)	0.11	0.36 (0.26 - 0.49)	0.63
	Q1-M (0. 12-<0.17)	1.04 (0.78 - 1.39)		0.48 (0.32 - 0.72)		0.51 (0.34 - 0.76)	
	M-Q3 (0. 17-<0.21)	1.05 (0.86 - 1.28)		0.47 (0.33 - 0.69)		0.38 (0.25 - 0.57)	
	>Q3 (>0.21)	0.59 (0.46 - 0.76)		0.30 (0.22 - 0.41)		0.40 (0.29 - 0.56)	

VCY (log_{10} cp * year/mL)	<Q1 (<0.94)	1.58 (1.09 - 2.30)	0.0017	0.97 (0.63 - 1.49)	<0.001	0.62 (0.36 - 1.07)	<0.001
	Q1-M (0.94 -<2.35)	1.04 (0.77 - 1.41)		0.66 (0.48 - 0.91)		0.54 (0.37 - 0.80)	
	M-Q3 (2.35 -<4.63)	0.71 (0.54 - 0.92)		0.40 (0.28 - 0.55)		0.67 (0.51 - 0.88)	
	>Q3 (>4.63)	0.62 (0.53 - 0.73)		0.22 (0.17 - 0.30)		0.25 (0.18 - 0.33)	

Table 6.12. Unadjusted and Adjusted Rate Ratios of the development of class of resistance according to VL exposures

		NRTI		NNRTI		PI	
		Adjusted RR	**GP[1]**	**Adjusted RR**	**GP[1]**	**Adjusted RR**	**GP[1]**
Start of the VF episode VL (copies /mL)	51-500	0.67 (0.30 - 1.51)	0.800	0.46 (0.16 - 1.31)	0.536	0.68 (0.32 - 1.47)	0.659
	501-1,000	0.89 (0.36 - 2.20)		0.78 (0.28 - 2.21)		0.50 (0.17 - 1.46)	
	1,000-5,000	0.96 (0.44 - 2.10)		0.71 (0.29 - 1.72)		0.72 (0.35 - 1.48)	
	5,001-10,000						
	10,001-	0.84 (0.39 - 1.79)		0.51 (0.20 - 1.33)		0.69 (0.35 - 1.35)	
	>100,000	0.91 (0.38 - 2.15)		0.76 (0.28 - 2.12)		1.17 (0.54 - 2.57)	
Average VL (copies /mL)	51-500	1.99 (0.65 - 6.10)	0.438	2.41 (0.29 - 19.79)	0.513	3.53 (0.81 - 15.43)	0.017
	501-1,000	2.35 (0.78 - 7.10)		1.77 (0.32 - 9.70)		1.77 (0.65 - 4.86)	
	1,000-5,000	1.60 (0.86 - 2.98)		1.21 (0.61 - 2.40)		1.55 (0.69 - 3.47)	
	5,001-10,000						
	10,001-	1.35 (0.77 - 2.38)		1.43 (0.77 - 2.64)		1.07 (0.53 - 2.16)	
	>100,000	1.05 (0.56 - 1.94)		0.80 (0.38 - 1.67)		1.27 (0.62 - 2.63)	
Peak VL (copies /mL)	51-500	2.11 (0.87 - 5.15)	0.439	1.51 (0.27 - 8.43)	0.468	2.77 (1.05 - 7.35)	0.556
	501-1,000	1.54 (0.70 - 3.39)		1.69 (0.56 - 5.08)		2.04 (0.52 - 8.00)	
	1,000-5,000	1.10 (0.67 - 1.83)		1.49 (0.73 - 3.05)		2.19 (1.03 - 4.66)	
	5,001-10,000						
	10,001-	1.25 (0.71 - 2.22)		1.60 (0.81 - 3.13)		2.59 (1.22 - 5.49)	
	>100,000	1.85 (0.88 - 3.89)		2.24 (1.04 - 4.82)		5.63 (2.51 - 12.59)	
VL slope (log_{10} cp/mL/year)	<Q1						
	Q1-M	2.95 (1.87 - 4.65)	<.001	2.70 (1.48 - 4.93)	0.033	2.65 (1.41 - 4.99)	0.018
	M-Q3	2.15 (1.39 - 3.32)		1.38 (0.65 - 2.92)		2.11 (1.14 - 3.93)	
	>Q3	1.17 (0.73 - 1.86)		1.24 (0.68 - 2.27)		1.75 (1.00 - 3.05)	

VCY (log_{10} cp * year/mL)	<Q1						
	Q1-M	0.52 (0.26 - 1.08)	<.001	0.54 (0.27 - 1.07)	<.001	0.49 (0.19 - 1.23)	<.001
	M-Q3	0.15 (0.08 - 0.29)		0.06 (0.03 - 0.12)		0.25 (0.10 - 0.61)	
	>Q3	0.03 (0.01 - 0.06)		0.01 (0.00 - 0.02)		0.02 (0.01 - 0.06)	

1. GP=Global P

6.5. Discussion

In this analysis I found that resistance mutations accumulated at a rate of 1.51 (1.37-1.68) new mutations per year in individuals being kept on a failing regimen. This is equivalent to one new mutation accumulating every 7 months and 1 drug option being lost every 24 months of a patient being kept on a failing regimen. This rate was lower when only considering mutations associated with a reduction in susceptibility to the drugs included in the failing regimen. The overall rate of resistance accumulation was slightly lower than in prior EuroSIDA analyses (356), but in line with recent estimates from the PASER-M and MARCH cohorts (1.45 new mutations/year) and an analysis by Napravnik et al of patients in North Carolina (1.61 new mutations/year) (645,646). However, the rate of NNRTI resistance accumulation was low compared to the analysis of the PASER-M data by Boender et al (645). This could reflect the relatively low proportion of individuals receiving NNRTI-based cART in my analysis.

Resistance accumulation was borderline associated with VL at the start of episodes in univariable analyses, but this association was attenuated and no longer significant after controlling for HIV subtype and CD4 counts and GSS at start of the VF episode. This is consistent with earlier findings by Napravnik et al, who also found a univariable association between start of the VF episode VL measures and resistance development which was no longer significant after adjusting for potential confounders (646). It is also in agreement with findings by Adjé-Touré et al from a cohort of people recruited in Cote D'Ivoire (658) and Bangsberg et al (657), who both found that VL at the start of the VF episode was not associated with the risk of detection of resistance, at least when modelled linearly. However, the results are conflicting with those from other cohorts which have found both higher (355) and lower (486) risks of resistance detection with increasing VLs when considering VL at the start of the VF episode as a predictor. As mentioned in the introduction results from different studies looking at this question are often difficult to compare, particularly if the authors have included baseline viral load as a continuous variable. I decided *a priori* to include VL at the start of a VF episode as a categorical exposure. Although, given the body of existent evidence, the categorical approach is more likely to approximate the true underlying shape of the relationship between VL at the start of VF and risk of resistance accumulation, the choice of the categories can impact the estimated relationship. I chose categories that related to thresholds that have been used be the WHO to define VF and therefore have some clinical meaning.

Out of all the VL exposures considered, the rate of resistance accumulation was independently associated with average VL during an episode, change in VL during an episode and VCY after adjustment for potential confounders. Regarding the average VL during the episode, I found

that those individuals with low to intermediate (5,001-10,000) average VLs were the least likely to show an accumulation of resistance. My findings contrast with those of Napravnik et al, who instead found that the risk of accumulating new mutations was the greatest among patients with similar average VL intermediate levels (646). Possible explanations for this discrepancy include differences in the case mix of patients (such as different patients receiving different regimens at different stages of HIV disease. It is also possible that the frequency of VL measurements impacted on the construction of the exposure variable. Reassuringly however, low levels of resistance accumulation was found at the highest levels of average VL both in this analysis and in the analysis by Napravnik et al after adjustment (646). No other studies included in my literature review evaluated the association between a summary measure of the average VL levels over a period of virological failure and the risk of resistance development.

The relationship between estimated change in VL values (VL slope) and resistance accumulation appeared to follow an inverse U-shape in my analysis. Individuals with intermediate VL slope values (0.12-<0.21 log_{10} cp/mL/year) had the highest rates of resistance accumulation, and accumulation appeared to be lower among those with VL trajectories that either changed very little (<0.12 log_{10} cp/mL/year) or those that changed quite markedly (>0.21 log_{10} cp/mL/year). These results seem to fit with a priori speculative thinking regarding the association between level of viral replication, adherence to therapy and risk of accumulating resistance. First of all, individuals with low VL slope values may be at lower risk of accumulating resistance because they are those not experiencing high enough levels of viral replication for mutation selection to occur. On the other hand, as has been previously hypothesised, individuals with sharp increases in the VL may have adherence issues leading to an absence of selective pressure (646,664). Napravnik et al also studied how changes in the VL slope during VF relate to the risk of resistance accumulation. They found that accumulation was lower when the VL slope was low or stable (<0.2 log_{10} copies/ml/year) and higher when the VL slope was high (>0.2 log_{10} copies/ml/year) (646). Although this differs slightly from the findings of my analysis in which resistance accumulation was relatively low at VL slope values of 0.21 log_{10} copies/mL or higher, the categorisation of the VL slope variable and choice of comparator group are likely to influence this comparison. Other studies have also assessed whether VL changes during a period of VF affects the rate of resistance accumulation (356,357,652) . Kantor et al, when analysing 106 patients receiving care at Stanford University Hospital, did not find any evidence that changes in the VL affected the rate of resistance accumulation markedly (357). Cozzi-Lepri et al, analysing data from the EuroSIDA cohort, found that a greater change in VL during the FU time was marginally associated with a greater accumulation of drug resistance mutations (356), although the investigation of this

relationship was not the primary objective of the analysis and therefore details of the size of the effect or modelling approach were not published. Hanson et al, analysing data from a cohort of people from the Cote d'Ivoire national drug access programme found that the lower the size of the VL decline after the start of treatment (not necessarily during VF), the lower the probability of detecting resistance (652). However, this analysis included individuals who were not experiencing VF rather than people on a stable failing regimen so their findings are not directly comparable with mine.

VCY also showed a strong association with the risk of accumulation of resistance, with the rate of resistance accumulation decreasing markedly with increasing levels of VCY, with a maximum rate of accumulation of 3.8 new mutations/year seen in those with the lowest VCY group (<0.94 $\log_{10}$ cp * year/mL). Using pre-specified cut-offs for the categorical variable, the rate appear to decrease in a linear fashion. One potential explanation of this finding is that VCY acts as a proxy for selective pressure, with increasing VCY being indicative of poor adherence when properly accounting for the length of the episode, the actual exposure to different levels of replication or both.

In terms of clinical relevance, the finding with perhaps the strongest policy implications is the lack of an association between VL at the start of VF and the consequent accumulation of resistance. This might, at least indirectly, indicate that VL levels at the start of failure used in isolation may not be an appropriate measure to determine whom, amongst people experiencing VF, to switch to another treatment immediately without waiting for a confirmatory VL and whom to target for adherence interventions. In addition to the lack of a significant association, mutations were found to still accumulate among those individuals whose failure VL was in the lowest 51-500 copies/mL category, consistent with previous findings (565). This suggests that there is no VL failure threshold below which resistance accumulation is negligible, and supports recent WHO guidelines lowering the threshold for the definition of VF in low income settings from 5,000 to 1,000 copies/mL (72).

My analysis did not consider predictors of resistance accumulation apart from VL in detail, as risk factors for resistance was investigated as a main aim of Chapter 3. Nonetheless, some findings from other studies were consistent with those shown by the univariable analysis. This included the findings of lower resistance accumulation rates among individuals with non-B viruses (336), among those receiving a boosted PI (356,565,656) and among those with lower CD4 counts (649,656,665). I also found a higher rate of resistance accumulation with longer duration of treatment failure. This is in broad agreement with previous findings (357,486,649,651), although, as extensively discussed for specific predictors above, risk factors

for resistance accumulation are likely to vary somewhat according to the populations studied. Perhaps surprisingly, mutations appeared to accumulate faster at lower values of GSS in this analysis. This is in contrast to reports from prior studies (356,357), but, again, this is likely to be explained by differences in the populations studied. In an ideal world of perfect adherence to treatment, people with high GSS are those with little resistance being present and great chance of suppressing viral load given the large number of active options available. However, in the context of people failing regimen and remaining on such a regimen for some time, a high GSS is more likely to be associated with people whose lack of resistance is explained by non-adherence. People with low adherence on a failing regimen are also expected to accumulate more resistance. Indeed, I found low rates of resistance accumulation among individuals with no detected resistance at the start of an episode (0.16 new mutations/year), providing some support for this hypothesis.

6.5.1. Strengths and limitations

This study has a number of strengths and limitations. In terms of strengths, it is the largest study to date to describe the accumulation of resistance in relation to VL exposures using repeated resistance tests. The clearly defined VL exposures and transparent modelling also add to the strengths of the analysis. However, there are also a number of limitations. Firstly, the individuals included experienced VF in very early calendar years and received regimens that would no longer be considered as first line treatment, even in low-income settings. This could limit the generalizability of the findings. However, the rate of accumulation of resistance found here was remarkably similar to that found in a much more recent analysis of individuals failing first line contemporary treatment (645). It was not possible to conduct a sensitivity analysis restricting the analysis to more recent calendar years, as the number of individuals experiencing VF with repeated resistance tests in recent calendar years was too low in EuroSIDA. Although the relationship between VL and resistance accumulation can partly be explained through biology, it is clearly also affected by behavioural factors such as adherence to therapy. The lack of a validated measure of adherence in this dataset is a significant limitation, as any of the hypothesised explanations for the shape of the relationships found which are related to adherence to treatment cannot be directly be tested. In order to maximise the number of people that could be included in this analysis I assumed that those starting their first ART regimen from ART-naïve and for whom a resistance test before initiating ART was not available, did not have any transmitted drug resistance. It is therefore possible that some of the resistance detected during failure were already present at baseline and were not accounted for. Finally, the methodology applied here implicitly assumed that resistance mutations accumulated at a stable rate until a GRT was done or results of the test recorded,

allowing for an accurate estimation of FU time. This is unlikely to be the case, and the use of the date of the last GRT to determine the end of the failure episode could lead to an under-estimate of the true rate of accumulation over the episode (e.g. if mutations have emerged but not yet taken over the majority circulating virus and therefore remain undetectable by Sanger sequencing at the time of the GRT) or an over-estimate of the true rate (if mutations emerged long before the GRT was done so that too many person years are included in the denominator). Additional limitations of EuroSIDA is the relatively low prevalence of people enrolled when ART-naïve and the low prevalence of people harbouring non-B strains of HIV, although the latter is a limitation shared by most of the European cohorts of HIV-infected individuals. It is reassuring that cohorts in other settings found similar rates of resistance accumulation to those I estimated (645).

6.5.2. Conclusion

In conclusion, I have estimated that individuals maintained on a failing treatment regimen accumulate approximately 1 new mutation per every 7 months and lose 1 drug option for every 24 months of being maintained on the failing regimens. The lowest level of resistance accumulation is likely to occur among individuals whose VL changes less than 0.12 $\log_{10}$ copies/ml/year and those with average VL values between 5,001 and 10,000 copies/mL. This seems to be consistent with what is known about the viral biology, as it is expected that resistance accumulates less in people with impaired viruses replicating at low levels. However, individuals with steeper VL increases (more than 0.21 $\log_{10}$ copies/mL/year) or high VCY values are also likely to experience low resistance accumulation. Although it is a hypothesis that could not be tested, the most likely explanation for this is poor adherence in those with a history of high level of high replication. Another key result is the lack of an association between VL at the start of a failure episode and the risk of resistance accumulation, possibly suggesting that the choice of maintaining a patient on a failing treatment regimen should take into account factors besides the level of viral load observed at first detection of viral failure.

Although the aim of current antiretroviral therapy should always be to construct a fully suppressive regimen, there is a small proportion of people currently receiving care in clinics across Europe for whom this is not achievable because they have developed resistance to all drug classes of antiretrovirals, including integrase inhibitors. These findings provide important data on the rate of resistance accumulation to three historical drug classes should maintenance therapy be necessary. In addition, it provides insights into the rate of resistance accumulation that could reasonably be expected according to a wide range of measures of VL exposures each focussing on different aspects of viral load replication history. Future studies investigating the relationship between viral replication and resistance accumulation should

ideally include validated measures of adherence. Specific questions for future research, as well as potential alternative study designs, are discussed in more detail in Chapter 8.

6.6. Dissemination of results

Some of these results were presented at the 2015 International HIV Drug Resistance Workshop in Boston, and was awarded with the Chairman's Poster Prize (Appendix IX). A manuscript is in preparation.

Chapter 7 . The effect of primary drug resistance on CD4 cell decline and viral load set point in HIV positive individuals before the start of ART

7.1. Introduction and Objectives

Chapter 5 and 6 described the impact of maintaining individuals on a failing treatment regimen on CD4 counts and resistance development. However, these are not the only concerns associated with the use of maintenance antiretroviral therapy (ART). Individuals with high VLs are more likely to transmit the virus (62,666), and maintaining individuals on ART despite the presence of on-going viral replication could therefore contribute to the spread of drug resistant strains. Transmitted drug resistance (TDR) has been associated with an increased risk of first line ART failure following the start of ART unless genotypic resistance testing (GRT) is used to construct a regimen that is predicted to be fully suppressive (373). However, it is also possible that the presence of TDRM or other genetic changes in the viral genome influence disease progression before the start of ART. Differences in fitness between viruses carrying different types of drug resistance mutations (DRM) and wild-type strains could result in differences in pathogenicity, and thus influence both the natural history and potential spread of HIV (375). DRM that strongly affect fitness have been speculated to result in lower set point viral loads and higher CD4 cell counts, and consequently a slower disease progression (667). However, it is also possible that DRM associated with low fitness costs or fitness increases could lead to an increased CD4 decline followed by a more rapid disease progression (375,379) as well as greater viral transmission potential (668). Genetic changes affecting such parameters are unlikely to be restricted to mutations commonly classed as evidence of TDR, as treatment associated polymorphic or compensatory mutations have also been shown to have an effect on the fitness of viral strains (669).

Although all individuals should start treatment as soon as possible after being diagnosed with HIV (601,670,671), any such differences could influence both clinical outcomes among undiagnosed HIV-infected patients and the transmission dynamics of the HIV epidemic on a population level (669,672).This has important implications for mathematical models of the disease and consequently the development of public health policies (672,673). Previous research has found that the detection of any TDRM can lead to a more rapid disease progression in the first year after infection (375), but the impact of specific mutations has rarely been comprehensively evaluated. The aim of this analysis was therefore to investigate

the effect of resistance mutations on the viral load set point and CD4 count decline before the start of ART.

The specific objectives for this analysis were:

1) Compare CD4 changes among individuals with either any resistance, class-wide resistance or any single mutation to that among individuals infected with a virus without resistance.

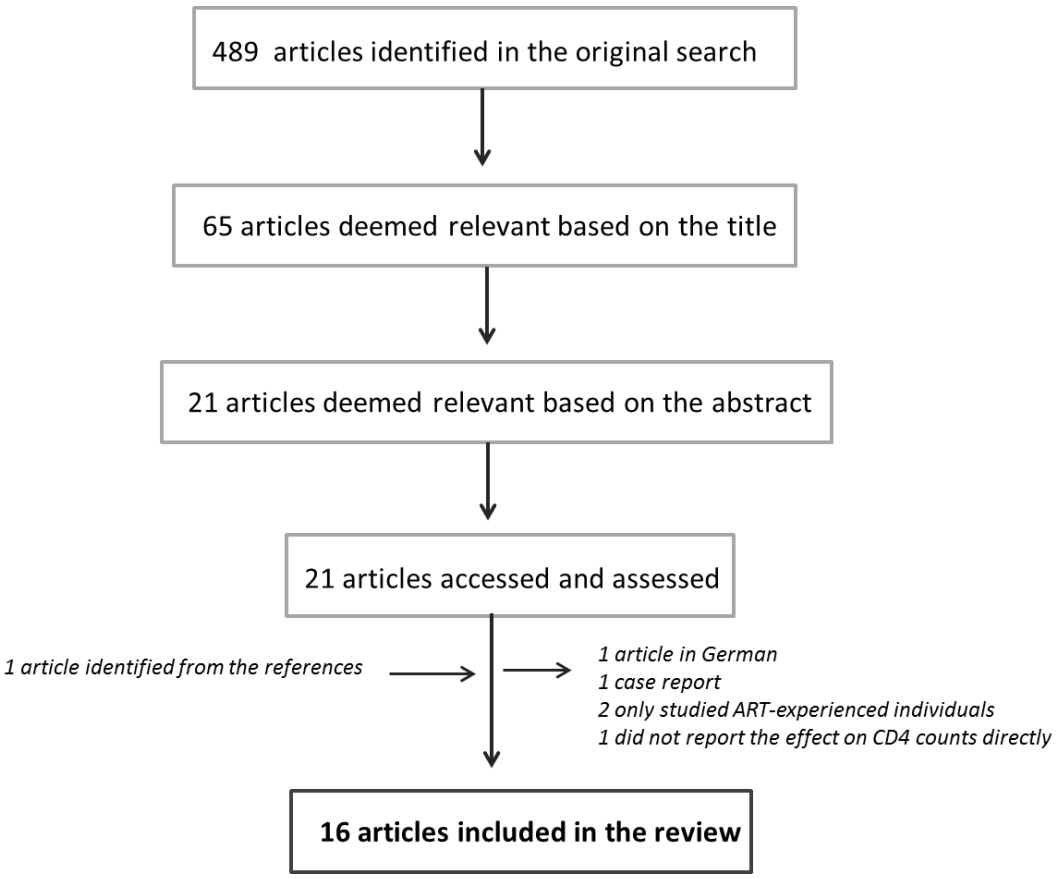
2) Compare the VL set point among individuals with either any resistance, class-wide resistance or any single mutation to that among individuals infected with a virus without resistance.

3) Conduct a principal component analysis (PCA) in order to identify clusters of mutations, and evaluate the effect of reverse transcriptase (RT) and protease (PR) clusters on the CD4 count decline and VL set point through the use of extracted component based scores.

7.2.Literature Review

I conducted a literature review in order to identify papers which have previously evaluated the association between resistance and CD4 counts and/or the viral load before the start of ART. In total, I could identify 16 papers (Figure 7.1). These are described in detail in Table 7.1, and the findings are briefly summarised below.

Figure 7.1. Identification of articles for inclusion in the literature review



7.2.1.The effect of TDRM on CD4 counts and VL

The majority of the studies (N=12) investigated the effect of the presence of any TDRM on CD4 counts or the VL set point at a single point in time. In terms of CD4 counts, a significant number of studies failed to find an association between CD4 counts and any detected resistance (669,674–678). However, there were also studies reporting both positive and negative associations. A study by Karlsson et al of 1,491 newly diagnosed individuals in Sweden found that individuals with higher CD4 counts were less likely to have TDRM (679). This is in broad agreement with the results from a study by Huang et al, who analysed the data from patients receiving care in the Free State, South Africa. However, while Karlsson et al found an association between CD4 counts and resistance classed as transmitted by the WHO surveillance list, Huang et al only found an association between CD4 counts and the detection of treatment-associated resistance as defined using the Stanford system. In the analysis by Huang et al, the association between the detection of resistance and CD4 counts grew weaker when using the IAS list, and was no longer significant when using the WHO TDRM surveillance list to classify resistance (680). This indicates that it is important to distinguish between TDRM and treatment-associated resistance, as the classification system used may influence the findings. While Karlsson et al and Huang et al found that TDRM and treatment associated resistance mutations respectively were less likely to be detected at high CD4 counts, reasonably large studies from the UK (375), France (681) and the US (682) have all found that individuals with higher CD4 counts are more likely to have detected resistance before the start of ART. Differences in the resistance lists, study designs and study populations could be responsible for these discrepancies, although it is also possible that the class and extent of resistance present affect the findings. This would be in agreement with findings by Poon et al. They studied associations between Stanford scores for different classes of mutations and CD4 counts using data from the observational CFAR network in North America (683). While lower scores for NRTI and NNRTI mutations on the Stanford scale were both associated with higher CD4 counts, no association was found between PI scores and CD4 counts.

Two studies from the UK register on seroconverters studied the effect of TDRM on CD4 count changes over time (375,684). Bhaksharan et al conducted a preliminary analysis of 1,533 individuals in 2004 and found that CD4 decline in the first year following infection appeared to be faster among individuals infected with TDRM compared to those with no detected TDRM or no pre-ART resistance test (684). However, this difference was not statistically significant, and there was no evidence for a difference after the first year. The analysis was repeated in 2006, and restricted to those individuals with known resistance test results (N=300). This more

recent analysis confirmed a faster CD4 decline among individuals with TDRM in the first year of infection (375). An analysis of 424 individuals from the German HIV-1 seroconverter study also showed that individuals infected with TDRM experienced a more pronounced CD4 cell loss in the first year of infection. However, these individuals also had overall lower VL levels compared to individuals without TDRM, and neither of the differences were statistically significant (676). One analysis, by Bezemer et al, classified the HIV genomes of infected individuals as "evolving" if they showed genetic changes as detected by repeated genotyping. Although the sample size was small (N=20), they found that individuals infected with evolving HIV viruses experienced slower CD4 declines compared to those infected with strains that were not evolving. The authors suggest that an evolving or changing HIV genome may be a marker of low fitness, as more changes are expected in viruses with impaired replication capacity, which in turn could correlate with slower CD4 declines (685).

In terms of viral load, again, most studies report no association with drug resistance (669,674–676,686). A small number of studies reported that individuals with higher viral loads were less likely to have detected resistance (681,682). Harrison et al, analysing data from the UK drug resistance database, found that although the detection of any resistance was not associated with VL in either univariable or multivariable analysis, individuals with multiclass resistance or the M184V mutation had lower VL levels (686). In agreement with these findings, Poon et al also found that any amino acid change at position 184 of RT region was associated with a lower VL at baseline. When studying classes of resistance, Poon et al also found that higher Stanford scores for NRTI resistance was associated with lower VL levels, but higher scores for NNRTI resistance was associated with higher VL levels (683).

Only one study looked at the impact of patterns of mutations on CD4 counts/the viral load. Theys et al constructed fitness landscapes to derive fitness values representing the relative fitness of a given genotype compared to that of a HXB2 reference strain. Although no associations with any TDR or any individual treatment associated mutations and VL/CD4 counts were found, higher estimated fitness resulting from genetic variability in the PR (but not RT) was significantly correlated with both higher VL and lower CD4 count, most likely due to the presence of polymorphic and compensatory mutations in the PR gene (669).

7.2.2. What this analysis adds

The majority of the studies identified for this review focused on studying associations between TDRM and both CD4 counts and the VL set point at a single-point in time; with considerably fewer studies looking at repeat CD4 measurements and assessing CD4 changes over time before the start of therapy. Most of the identified studies were also relatively small, with 8/16 including fewer than 500 participants and very few studying the effect of individual mutations or the combined effect of clusters of mutation. Because of the inconsistent results identified in this literature review, the true role of TDRM and treatment associated compensatory mutations in the natural history of HIV remains largely unclear. This dataset, which comprises repeated viral load, CD4 count and genotypic test results in absence of antiretroviral treatment from HIV-infected individuals seen for care in a large number of European clinical centres offers a good opportunity to re-evaluate these questions using a much larger sample size than those previously available.

Table 7.1. Papers reporting on the effect of resistance on CD4 counts/the VL before the start of ART					
Author	**Year**	**Design & Setting**	**N**	**Main Results**	**REF[1]**
Theys et al	2012	Cross-sectional analysis of data from SPREAD, a European surveillance programme	1782	No association between TDR and CD4 counts or VL at baseline (p=0.14 and p=0.52, respectively). Adjustment for duration of infection or demographic variables did not change this. However, higher estimated fitness from a fitness landscape for PR was correlated with higher VL and lower CD4 count. No individual TDRM were found to be associated with VL or CD4 counts. However, a higher number of polymorphic mutations in the PR were associated with a higher viral load, a lower CD4 count and higher estimated fitness. No trends for RT polymorphic mutations	(669)
Karlsson et al	2012	Longitudinal analysis of trends in TDR prevalence in Sweden; also described factors associated with TDRM	1491	Patients with higher CD4 counts were less likely to have TDRM in multivariable logistic models adjusting for subtype and MSM. However, patients with and without TDR had similar median CD4 and RNA plasma levels at baseline.	(679)
Youmans et al	2011	Cross-sectional analysis of TDR prevalence in a cohort of newly diagnosed individuals in South Carolina	1277	No association between CD4 counts, viral load and TDRM. There were also no associations between the median first CD4 count and TDRM, but some evidence that the median first viral load was lower among individuals with TDRM.	(678)
Poon et al	2011	Cross-sectional analysis using data from the CFAR network, an observational cohort from North America	14111	Increasing Stanford scores for NRTI and NNRTI mutations were associated with decreasing CD4 counts. Increasing Stanford scores for NRTI were associated with decreasing VL as well, but increasing NNRTI scores were associated with increasing VL. Stanford PI scores were not associated with baseline CD4 or VL. A number of individual mutations, including D67X and K219X, were associated with lower baseline CD4 counts. M184X was associated with lower VL at baseline, as was	(683)
Huaman et al	2011	Retrospective study of 501 individuals newly diagnosed with HIV at the Henry Ford Hospital in Detroit from 2006-2008.	501	Resistance mutations were more likely to be detected at higher CD4 counts, and less likely to be detected at higher VL values.	(682)

Harrison et al	2010	Analysis of patients in UK CHIC and the UK HDRD.	7994	Presence of resistance to a single drug class was not associated with VL in either univariable or multivariable analysis. However, patients with multiclass resistance had lower mean VL. No other associations found for individual mutations, apart from M184V which was associated with lower baseline VL. It appeared that the effect of multiclass resistance on VL was mediated completely through M184V.	(686)
Huang et al	2009	Cross-sectional analysis of patients receiving care in the Free state province of South Africa in 2006	884	Drug associated polymorphisms were concentrated among patients with low CD4 counts when considering Stanford mutations; however, this association was only borderline significant when considering IAS mutations only (p=0.055) and even less so when only considering the WHO TDRM list (p=0.086). Authors speculate that this is probably the result of prior, undocumented drug exposure.	(680)
Peuchant et al	2008	Analysis of recently infected patients from the Aquitaine cohort in France, 1996-2006.	295	Baseline CD4 counts (at the start of ART) were higher in the resistant compared to the susceptible group (560 v 438 cells/mm^3, p=0.04). Baseline VL was significantly lower in the resistant group compared to susceptible (p=0.002).	(681)
Payne et al	2008	Cross-sectional analysis of resistance profiles from an audit of clinics in Yorkshire and the north-east of England	392	No associations found between TDR and baseline CD4 or VL.	(677)
Poggensee et al	2007	Analysis from the German HIV-1 Seroconverter Study	424	Baseline median VL and CD4 count at the time of seroconversion did not differ between the individuals with susceptible and resistant HIV infection, but there was some indication that VL was higher among those with no TDRM when time of infection was determined very accurately. People with resistant viruses appeared to have lower VL over the first year, but also more pronounced CD4 cell loss, although these findings were not statistically significant.	(676)
Pillay et al	2006	Longitudinal analysis from the CASCADE collaboration	300	Patients with TDRM experienced faster CD4 declines compared to those without TDRM, but only in the first year after infection. After one year, no differences between the two groups could be identified. Baseline CD4 counts were higher among those with resistance.	(375)

Bezemer et al	2006	Longitudinal analysis from the CASCADE collaboration	20	Some evidence that CD4 counts declined slower among patients who had evolving HIV viruses compared to those who did not have evolving viruses (p=0.043). Infection with a low fitness TDRM virus may have some protective effect, despite consequent reversions to wild-type viruses.	(685)
Oette et al	2004	Cross-sectional analysis of individuals starting ART in Germany	184	No difference in CD4 counts or VL between people with and without resistance.	(675)
Bhaskaran et al	2004	Exploratory longitudinal analysis of UK register of HIV seroconverters	1533	Suggestion of CD4 decline in the first year that was quicker among individuals with TDRM, but this finding was not significant. There was a suggestion of higher estimated CD4 counts at seroconversion, which again was not significant. There was no indication of any differences in terms of CD4 counts between individuals with and without TDRM in later year.	(684)
Grant et al	2002	Cross-sectional analysis of patients admitted to a hospital in San Fransisco, California	226	Found no difference between the presence of TDRM and pre-ART VL measures, but TDRM were associated with higher CD4 counts.	(667)
Rubio et al	1998	Study of patients starting ART at a centre in Spain.	38	No difference in terms of CD4 counts or viral load between individuals with and without TDRM at the time of starting therapy.	(674)

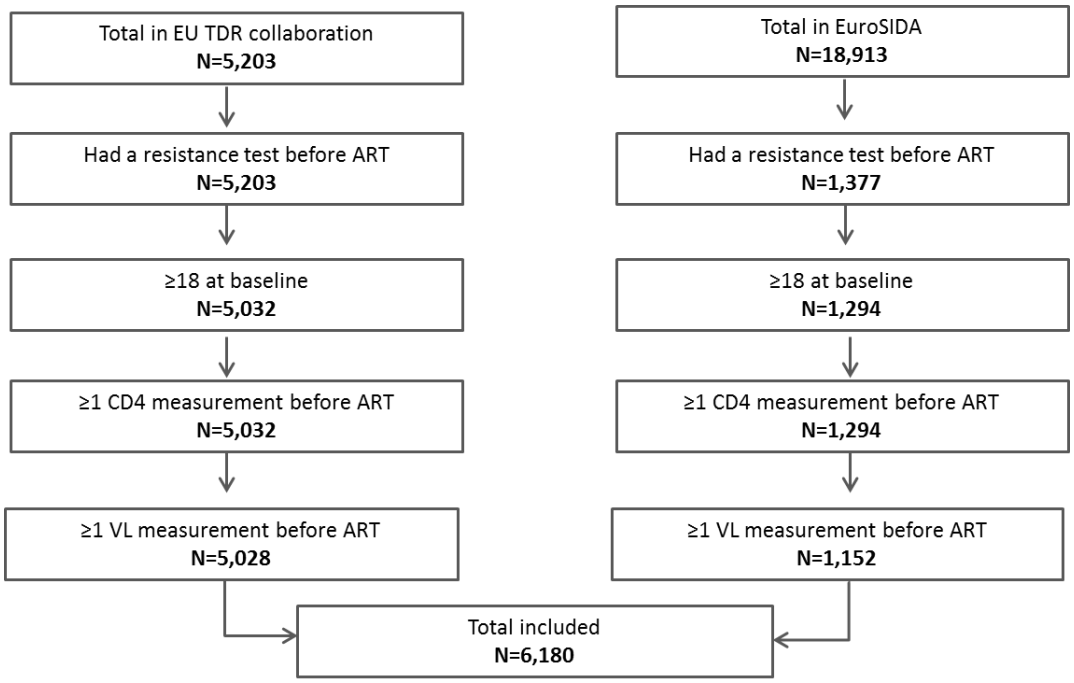
1. *REF=Reference*

7.3.Methods

7.3.1. Inclusion criteria

For this analysis, I used the D40 update of the EuroSIDA database and data from the EU-TDR collaboration. I included individuals over the age of 18 who had at least one resistance test and at least one CD4 count and VL measurement available before starting ART. In total, 6,180 individuals were included, and the selection process can be seen in Figure 7.1.

Figure 7.2. Selection of individuals for inclusion in the study



7.3.2. Resistance data

The classification of resistance present before the start of ART is complex. Because the literature review indicated that resistance classified according to the transmitted resistance surveillance list (687) and lists used to identify treatment associated resistance (394,662,663,688) might have a different impact on CD4 count and viral load, it was decided to study both resistance classically defined as TDRM using the WHO 2009 list (687), as well as drug resistance mutations (DRM) categorised according to the broader principles laid out in Chapter 5. Briefly, this involved using all four major resistance classification systems: ANRS, IAS, Stanford HIV database (HIVdb) and Rega. However, compensatory mutations, particularly in the protease (PR) gene, are also likely to influence the fitness of a given strain despite the fact that they may not directly affect drug-sensitivity. Therefore, non-polymorphic PR mutations associated with PI exposure were manually selected from the Stanford HIVdb according to the following rules:

(1) Non-polymorphic PR mutations with a prevalence of <1% among treatment naïve patients in the publicly available Stanford/HIVdb sequence database
(2) Non-polymorphic PR mutations with a prevalence of >1% among PI experienced patients in the Stanford/HIVdb sequences database
(3) Non-polymorphic PR mutations with a prevalence among PI experienced patients at least twice that among treatment naïve patients.

The aim of the first rule was to exclude polymorphic PR mutations. This is similar to the approach used to determine the WHO list of TDRM, but the 1% threshold makes it slightly broader than the TDRM list which uses a 0.5% threshold. This allowed the inclusion of a greater number of potentially compensatory PR mutations. The aim of the second and third rules was to identify mutations likely to appear under the pressure of PI-based treatment. Mutations meeting (1), (2) and (3) were considered potentially compensatory. Finally, I included a number of polymorphic substitutions in the PR gene that have previously been associated with fitness and disease progression (669). This selection process resulted in a list of 129 reverse transcriptase (RT) and 147 PR substitutions. Of these, I consequently evaluated 41 which met a pre-specified prevalence threshold in our dataset (1%) for their effect on CD4 counts and the VL (LL10I, L10V, T12N, I13V, I15V, GG16E, K20I, K20R, L33V, M36I, M36L, K43R, D60E, I62V, L63P, H69Q, H69N, H69Y, AA71T, A71V, T74S, V77I, L90I, QQ92K and I93L in the PR and M41L, D67N, A98S, K101Q , K101R, K103N, V106, V118I, E138A, V179D, V179I M184V,

V189I, L210W,T215S and K219Q in the RT). This threshold was chosen for the same reason as in Chapter 5, as mutations that occurred at a lower frequency were considered unlikely to be of clinical significance.

CD4 decline and VL levels among individuals with any TDRM or DRM, drug class-specific TDRM or DRM and individual mutations were compared to that among individuals with no resistance, defined as no NRTI, NNRTI or major PI mutations mentioned in the WHO surveillance list or in either of the four interpretation systems. Drug resistance was presumed to be present throughout the duration of the follow-up, irrespective of at which point it was detected. If an individual had more than one resistance test present during their follow-up, their resistance was considered cumulatively.

I also studied the impact of mutation patterns by conducting a principal component analysis (PCA) to identify clusters of mutations following the strategies outlined both in Chapter 2 (100) and Chapter 6 (217). After identifying clusters and extracting related scores, these were dichotomized using the 3rd quartile (Q3) as a cut-off point. This allowed me to broadly categorise individuals into those whose mutation pattern was similar to that described by a given cluster ("Above Q3") and those whose mutation pattern was not similar to that described by the cluster ("Below Q3").

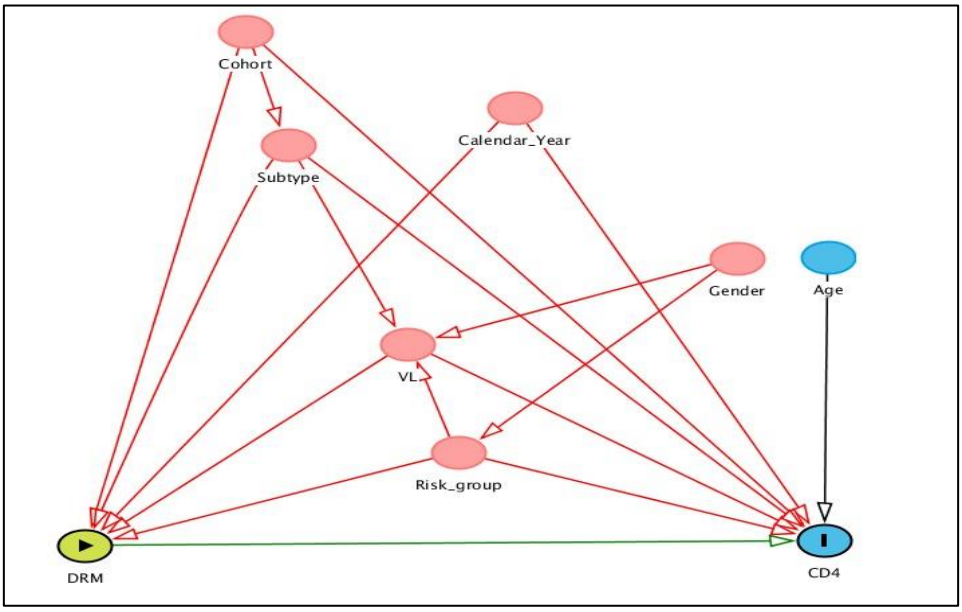
7.3.3. Statistical methods

Characteristics of the included individuals were compared according to the detection of TDRM and DRM using the chi-squared test and Kruskal Wallis test as appropriate. I used linear mixed models with a random intercept and slope to estimate the effect of resistance on CD4 counts and the VL set point. CD4 decline according to the detection of resistance was estimated by including an interaction between time and an indicator variable for the resistance exposure in a mixed model using CD4 counts as the outcome. The effect of resistance on VL was estimated by considering the effect of resistance on the intercept from a mixed model using VL as the outcome. This estimates a mean VL level, which can be interpreted as the VL set point. The rationale for using only the intercept for the viral load outcome is due to the relative stability of viral load over the course of the natural history of HIV (689). As in Chapter 5, I corrected the false discovery rate using the Benjamini-Hochberg procedure to calculate q-values for the analysis of individual mutations, as these were not selected on an *a priori* basis.

7.3.3.1. Model building strategy

As for the previous chapters, I drew a DAG as a visual guide to the covariate adjustment (Figure 7.3). Potential variables included in the DAG were selected based on clinical judgement and previous publications, but restricted given the data available in the EU-TDR collaboration.

Figure 7.3. DAG for the effect of DRM on CD4 decline before the start of ART



Given that the assumptions of the DAG (no measurement error and no unmeasured confounding) are correct, adjustment for risk group, subtype, cohort, calendar year and VL would be minimally sufficient to estimate a total direct effect of DRM on CD4 decline before the start of ART. As in Chapter 5, I conducted sequential adjustments for both outcomes as follows:

- Model 1: Adjusting for HIV risk group, viral subtype, calendar year of genotyping and cohort;
- Model 2: Model 1 + additional adjustment for the first recorded CD4 measurement (VL outcome model) and the first recorded VL measurement (CD4 slope outcome model).

Cohort was included as a surrogate marker for expected demographic differences, such as ethnicity and hepatitis co-infections status, that was hypothesised to act as potential confounders but for which we did not have data.

7.3.3.2. Variable categorisation

The resistance related exposures were all entered in the models as binary variables, and separate models were constructed for each of them. The type and, where relevant, categorisation of covariates included in the models can be seen in Table 7.2.

Table 7.2 Covariates included in the model

Variable	Categories	Time-updated
Cohort	EuroSIDA, Not EuroSIDA	No
Mode of transmission	MSM, IDU, Heterosexual, Other	No
Subtype	B, Non-B	No
Calendar year of the (first) resistance test	<99, 00-01; 02-03, 04-05, 06-07; 06-07, ≥08	No
First Viral Load	Linear, per $\log_{10}$	No
First CD4 count	Linear, per 100 cells/mm^3	No

7.3.3.3. Exploratory and sensitivity analyses

I conducted an exploratory analysis stratified according to subtype status (B v non-B), as it was considered possible that the effect of the selected resistance exposure variables on disease progression could differ across subtypes. I formally evaluated interactions for those resistance variables found to be significant in the main analysis by including a three-way interaction between subtype, time and the resistance exposure (CD4 analysis) and by including an interaction between subtype and the resistance exposure (VL analysis).

I also conducted a sensitivity analysis to address the risk of duplicate individuals from different cohorts having been included. It is possible that some patients in the EU TDR collaboration were included in the EuroSIDA database, although most of the centres that contributed data to the EU-TDR collaboration did so before they started contributing data to EuroSIDA. Unfortunately, it was not possible to identify potential duplicates on the basis of local ID's or date of birth, as these variables were not available in the EU-TDR dataset. Linkage on the basis of year of birth, first CD4 count and first VL value gave very low matching rates, and it was not considered feasible to accurately link the datasets on the basis of the available variables. Instead, I conducted a sensitivity analysis where all data from EuroSIDA was excluded.

7.4. Results

7.4.1. Demographic and clinical characteristics of the study population

6,180 individuals met the inclusion criteria and were included in the analysis. The characteristics of these individuals according to the source cohort are shown in Table 7.3. The majority of individuals were male (77%), most had acquired their HIV through sex with another man (46%) and were infected with a subtype B virus (64%). Individuals contributed a median of 5 CD4 measurements (IQR=3-9) and 4 VL measurements (IQR=2-8) over a median of 1.4 (IQR=0.1-3.8) years. Most individuals (90%) had just 1 pre-ART resistance test, but 623 (10%) individuals contributed 2 or more resistance tests. The baseline median CD4 count was 420 (IQR=289-583) cells/mm^3, and the baseline viral load was relatively high at 4.5 $\log_{10}$ copies/mL (IQR=3.9-5.0). The VL set point as estimated from univariable models was 4.4 $\log_{10}$ copies/mL (95%CI=4.4-4.4), and CD4 counts declined with an estimated 54 (95%CI=-56;-52) cells/mm^3/year. There were some differences between individuals included from EU-TDR and EuroSIDA clinics. Individuals from EuroSIDA tended to be younger ($p<0.001$), contribute data from earlier calendar years ($p<0.001$), were more likely to be MSM and PWID ($p<0.001$) and were less likely to have available subtype data ($p<0.001$) compared to individuals from EU-TDR clinics. Individuals from EuroSIDA also tended to have more advanced HIV disease with higher first VL values (4.6 [3.9-5.1] v 4.5 [3.9-5.9], $p=0.01$) and lower first CD4 counts (372 [255-510] v 433 [300-595], $p<0.001$).

The characteristics also varied according to whether individuals were infected with a virus carrying at least one TDRM, at least one DRM or no resistance (Table 7.4). Individuals with TDRM were more likely to be male than individuals with other DRM or no resistance ($p<0.001$), and they were generally genotyped in an earlier calendar year period ($p<0.001$). Individuals with at least one TDRM were also more likely to be infected with a subtype B virus, whereas individuals with other DRM were most likely to be infected with a non-B virus ($p<0.001$). In this cross-sectional part of the analysis, there was no evidence to suggest that either the median first CD4 or VL measurement differed according to resistance status ($p=0.12$ and $p=0.40$ for CD4 and VL respectively).

Table 7.3. Characteristics of the study population, according to cohort

		Total N(%)	EU-TDR	EuroSIDA	P-value
Gender[1]	Male	4560 (77.3)	3646 (76.8)	914 (79.4)	0.057
	Female	1339 (22.7)	1102 (23.2)	237 (20.6)	
Age, years	Median, IQR	36 (30, 42)	36 (30, 43)	34 (29, 41)	<.001
Baseline Date	Median, IQR	08/05 (12/02-07/07)	10/05 (10/03—7/07)	12/02 (02/97-01/08)	<.001
Risk Group[2]	MSM	2689 (45.9)	2084 (44.3)	605 (52.5)	<.001
	PWID	635 (10.8)	456 (9.7)	179 (15.5)	
	Heterosexual	1911 (32.6)	1617 (34.4)	294 (25.5)	
	Other	623 (10.6)	549 (11.7)	74 (6.4)	
HIV subgroup	B	3976 (64.3)	3252 (64.7)	724 (62.8)	<.001
	Non-B	1471 (23.8)	1301 (25.9)	170 (14.8)	
	Unknown	733 (11.9)	475 (9.4)	258 (22.4)	
Laboratory	First RNA, cp/ml (Median, Range) [3]	4.5 (3.9, 5.0)	4.5 (3.9, 5.0)	4.6 (3.9, 5.1)	0.014
	First CD4, cells/mm^3 (Median, Range)	420 (289, 583)	433 (300, 595)	372 (255, 510)	<.001

1. 281 individuals had missing data on gender

2. 322 individuals had missing data on risk group

Table 7.4. Characteristics of the study population, according to resistance presence

		Total N (%)	At least one TDRM	Other resistance	No resistance	P-value
Gender[1]	Male	4560 (77.3)	491 (83.2)	1894 (73.7)	2175 (79.4)	<.001
	Female	1339 (22.7)	99 (16.8)	676 (26.3)	564 (20.6)	
Age, years	Median, IQR	36 (30, 42)	37 (30, 43)	35 (30,42)	36 (30,43)	0.05
Baseline Date	Median, IQR	08/05 (12/02-07/07)	12/04 (10/01-04/07)	09/05 (02/03-08/07)	08/05 (01/03-07/07)	<.001
Risk Group[2]	MSM	2689 (45.9)	316 (53.5)	1035 (40.6)	1338 (49.2)	<.001
	PWID	635 (10.8)	63 (10.7)	245 (9.6)	327 (12.0)	
	Heterosexual	1911 (32.6)	141 (23.9)	987 (38.7)	783 (28.8)	
	Other	623 (10.6)	71 (12.0)	284 (11.1)	168 (9.9)	
HIV subgroup	B	3976 (64.3)	474 (76.7)	1565 (58.1)	1937 (67.5)	<.001
	Non-B	1471 (23.8)	66 (10.7)	820 (30.5)	585 (20.4)	
	Unknown	733 (11.9)	78 (12.6)	307 (11.4)	348 (12.1)	
Laboratory	First RNA, cp/ml (Median, Range) [3]	4.5 (3.9, 5.0)	4.4 (3.8, 5.1)	4.5 (3.9-5.0)	4.5 (3.9-5.0)	0.40
	First CD4, cells/mm^3 (Median, Range)	420 (289, 583)	426 (280, 610)	420 (280-570)	422 (296-588)	0.12

1. 281 individuals had missing data on gender

2. 322 individuals had missing data on risk group

7.4.2. Resistance prevalence

The overall prevalence of TDRM and DRM was 10% and 54%, respectively. Transmitted NRTI resistance was the class of TDRM most commonly detected, at 7.1%. This was followed by NNRTI (3.2%) and PI (2.6%) resistance. Regarding DRM, PI resistance was most common (31.3%), followed by NNRTI resistance (25.3%) and NRTI resistance (11.1%). The combined prevalence of minor and major PR mutations was, as expected, very high at 95.2%. The prevalence of individual mutations can be seen in Figure 7.4. Overall, 41 mutations were present at a frequency higher than 1% and were evaluated in multivariable models (Figure 7.4). The most common mutations were PR mutations, with L63P present in 40.8% of individuals. The most common RT mutation was V179I (6.5%).

Figure 7.4. Prevalence of transmitted and treatment associated resistance

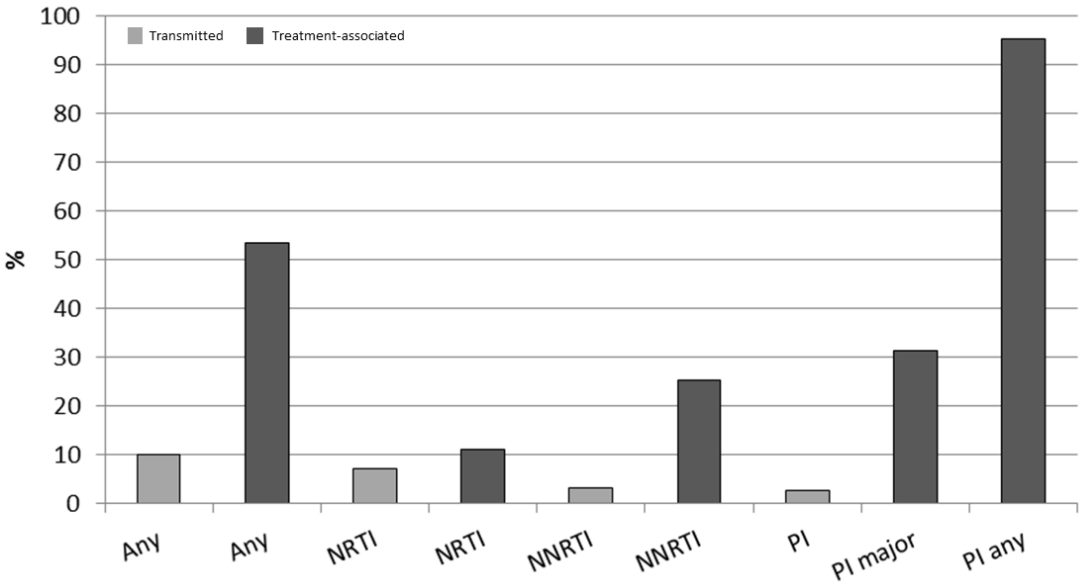
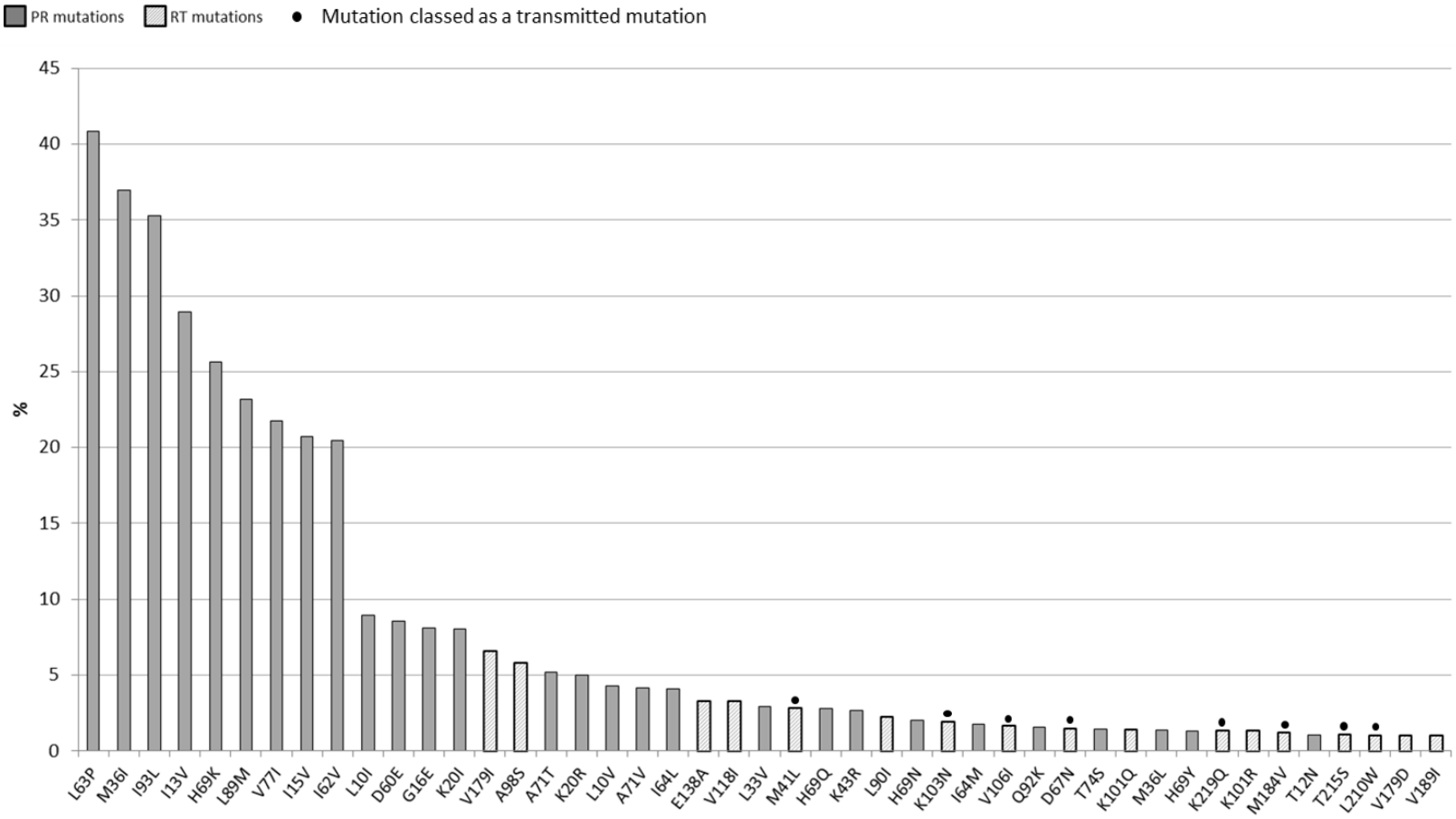


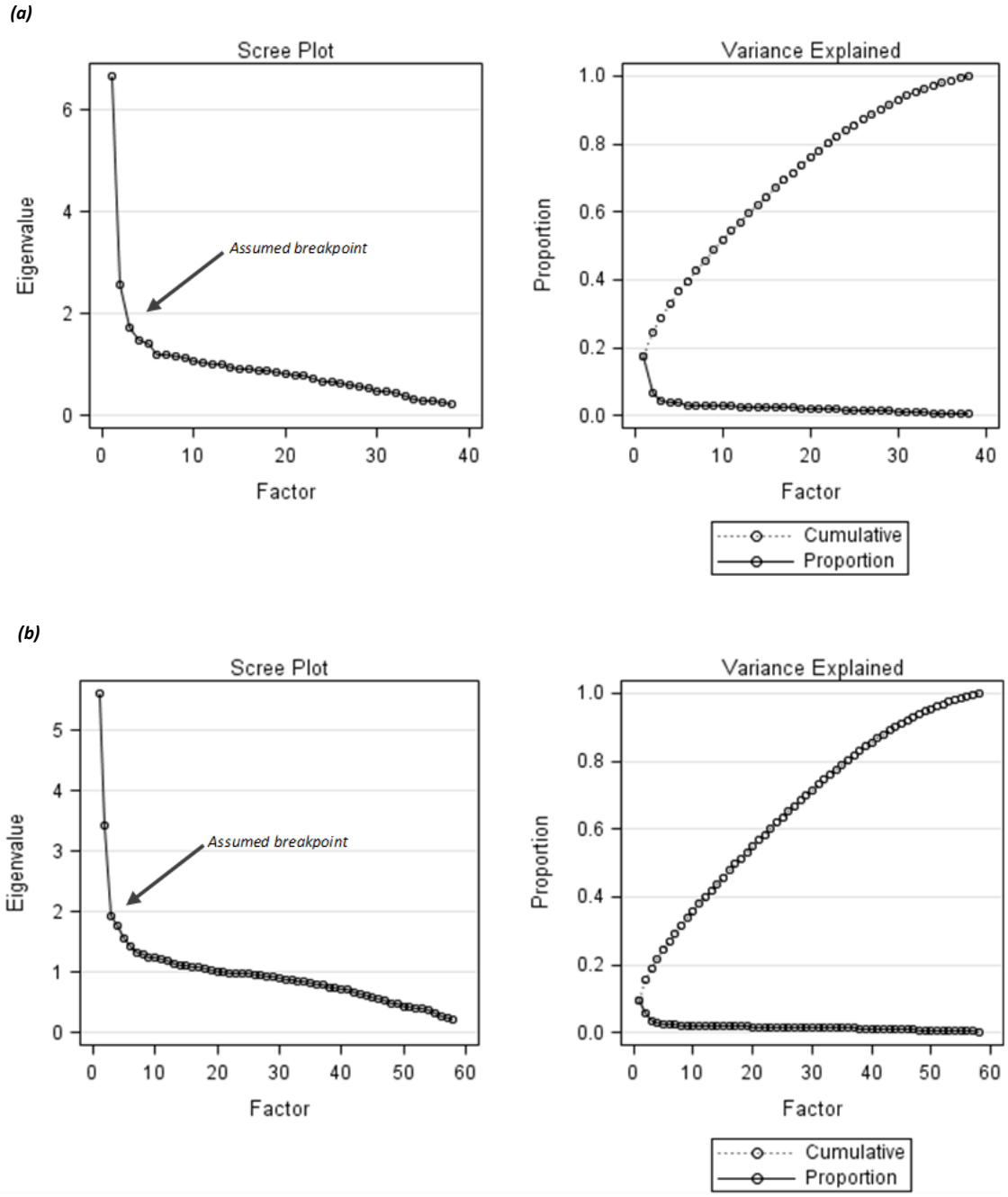
Figure 7.5. Prevalence of individual mutations at baseline present at a frequency of 1% or higher



7.4.3. Clusters of mutations

Following the principles laid out in Chapter 2, I identified two RT and two PR clusters through the use of PCA. The scree plots used to determine the number of components to retain can be seen in Figure 7.6a-b. Both the RT and PR clusters explained a low proportion of the overall variance, 24.3% and 15.6% respectively.

Figure 7.6. Scree plots for the RT (a) and PR (b) PCA



The contribution of each mutation to the extracted components, or clusters, can be seen in Table 7.5 and Table 7.6. The contribution, or loadings, in the tables can be interpreted as the standardised correlations between the resistance mutations and the unit-scaled components. For example, 116Y and 115F in RT had the highest correlation to the first cluster (loadings=83). The first RT cluster contained a large number of RT mutations that conferred both NRTI and NNRTI resistance. This included the 151M complex (substitutions in position 151, 115, 116 75, 77 and 62) together with substitutions in positon 74, 65, 100, 188, 179 and 230. The strongest contributions came from the mutations forming part of the 151M complex. The second RT cluster contained another previously well described RT cluster, the thymidine analogue mutations (TAM), and included substitutions in position 41, 67, 219, 215, 210 and 70 as well as a polymorphic substitution in position 44, the 184 substitution and a substitution in position 181 (Table 7.5).

The first PR cluster contained a number of major PI substitutions (position 46, 82, 47, 30, 32, 84, 48, 90, 50, 54 and 88) as well as a few minor PI mutations in position 73, 53 and 24. The second PR cluster contained 5 minor/polymorphic PR substitutions in position 20, 13, 36, 69 and 89 (Table 7.6).

Table 7.5. Loadings for the RT PCA[1]

	RT Factor 1 *Cross NNRTI/NRTI*	RT Factor 2 *Cross NNRTI/NRTI*
RT41L	8	61
rt103NSHTR	23	28
rt67NGESHT	14	67
rt219WHQNER	14	56
rt184VI	27	44
rt215SDYCFVIACEGHLN	6	67
RT210W	19	59
rt70RGQNSTE	27	41
rt190TASCEQV	32	32
rt101EPHIRQNT	13	18
RT225H	56	7
rt74VI	47	36
RT77L	80	-7
rt151ML	81	-6
rt65REN	58	7
rt100IV	54	20
rt75MTASIL	66	4
rt188LHCF	46	10
RT115F	83	3
RT116Y	83	-7
rt181CISFGV	32	40
rt230IVL	66	12
rt106AIM	18	16
rt179DLTIMEF	8	3
rt62V	45	-6
rt90I	1	3
rt98GS	12	7
rt108I	45	6
rt138AKQGRS	-1	4
rt221Y	0	15
rt44DA	31	41
rt40F	-2	11
rt118I	15	29
rt348I	0	-1
rt234I	0	0
rt238TN	-1	19
rt318F	0	-1
rt189I	0	-1
rt318F	0	-1
rt189I	0	-1

1. *Factor loadings below 30 were considered negligible, between 30-40 weak (light gray), above 40 strong (medium gray) and above 70 (dark gray) very strong. Mutations with loadings above 40 were interpreted as forming part of a cluster captured by that PC; those with loadings between 30 and 40 were considered weakly associated with the cluster*

Table 7.6. Loadings for the PR PCA[1]

	Component 1 ***Cross PI***	Component 2 ***Accessory non-B***
E34DQKV	0	1
K55NR	10	-6
T91A	3	5
K45QI	1	0
I66LVF	6	0
C67F	14	-1
L10YVCFIMR	21	5
K20LIMRTV	11	64
I13V	-1	57
D60E	-2	-14
I62V	5	-30
AA71TVI	26	-28
V77I	5	-39
I93ML	5	-26
L23I	9	-2
M46LIV	55	-4
V82TFSMACLG	64	-4
I47VA	72	3
I85V	13	6
D30N	46	0
V32IL	68	5
G73TCASVF	65	1
I84ACV	67	1
F53YWL	57	-1
I48VASTQLM	62	1
L90M	60	-3
I50VL	64	4
I54ATSMVL	67	1
L76V	8	1
N83D	8	4
L24IFM	59	1
N88DSTG	51	2
V11IL	4	3
GG16E	-3	34
L33FIVM	19	-9
M36ILV	4	76
K43TIR	7	-5
I15AV	4	15
L63PI	9	-40
H69KNQYIR	-1	75
L89RTFIMV	1	84
Q58E	3	0
E35GN	3	15
K70E	-1	-2
R41IT	0	1
T74ESA	3	15
QQ92KR	0	17
T12NKE	0	-1
Q61D	3	1
G17D	-1	0
Q18H	10	1
V75I	-1	0
N37Q	-1	-5
P79D	0	-2
A22V	7	5
C95F	15	-4
L38W	0	-1
I64MVL	0	-12

1. *Factor loadings below 30 were considered negligible, between 30-40 weak (light gray), above 40 strong (medium gray) and above 70 (dark gray) very strong.*

7.4.4. Effect of resistance on CD4 cell decline

7.4.4.1. Associations between TDRM, DRM and CD4 decline

The CD4 cell decline was estimated to be 53 cells/mm^3/year (95%CI=-56; -49) among individuals infected with viruses without resistance and 55 cells/mm^3/year (95%CI=-63; -48) among those infected with a virus carrying any TDRM (p-value for difference=0.47). These estimates did not change markedly upon covariate adjustments (Figure 7.7a). There was also no evidence that the detection of TDRM according to specific drug classes was associated with reduced or increased CD4 declines (Figure 7.7a). The findings were similar when considering DRM rather than TDRM (Figure 7.7b), although there was slightly stronger evidence suggesting that the detection of NNRTI DRM was associated with steeper CD4 decline still compared to people with no resistance (difference = -6 cells/mm^3/year, 95% CI=-12;0, p=0.04 after adjustment, Figure 7.7b).

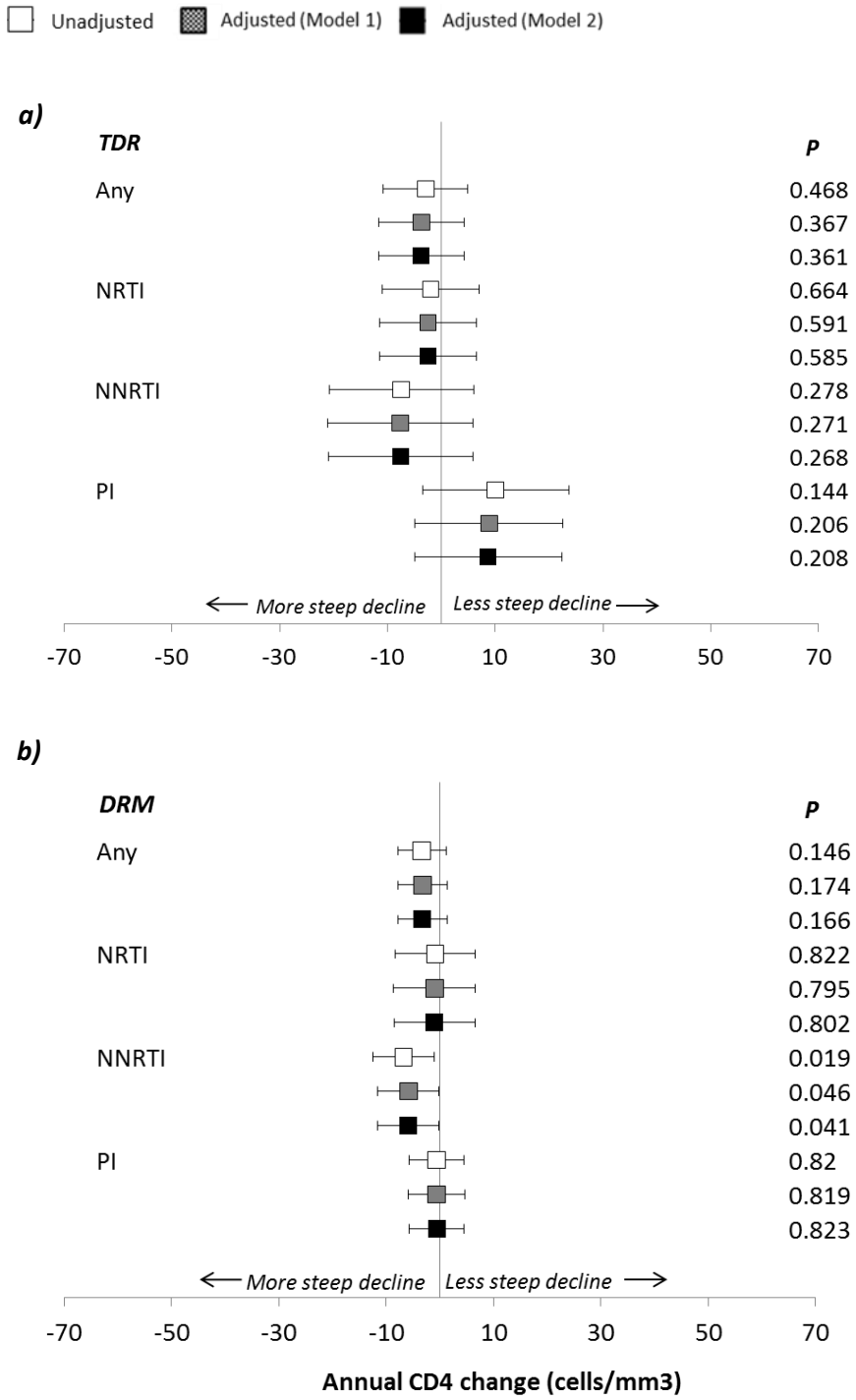
7.4.4.2. Associations between individual mutations and CD4 decline

No individual mutation was associated with the CD4 slope (Figure 7.8). The strongest signals were found for the A71T and L10V substitutions in PR gene and K101Q in the RT gene, which were associated with a steeper CD4 decline albeit not significantly after correcting for multiple testing. The largest difference was found for K101Q. Individuals with this mutation experienced a CD4 count decline of 26 cells/mm^3/year (95%CI=-45; -6) greater than those with no resistance detected. Individuals with the A71T or L10V mutation had CD4 counts which declined with around 16 cells/mm^3/year faster (-17 [-29; -4] and -16 [-27; -2] respectively) compared to individuals with no resistance detected. Adjustment for VL did not markedly affect the estimates (Figure 7.9).

7.4.4.3. Associations between clusters of mutations and CD4 decline

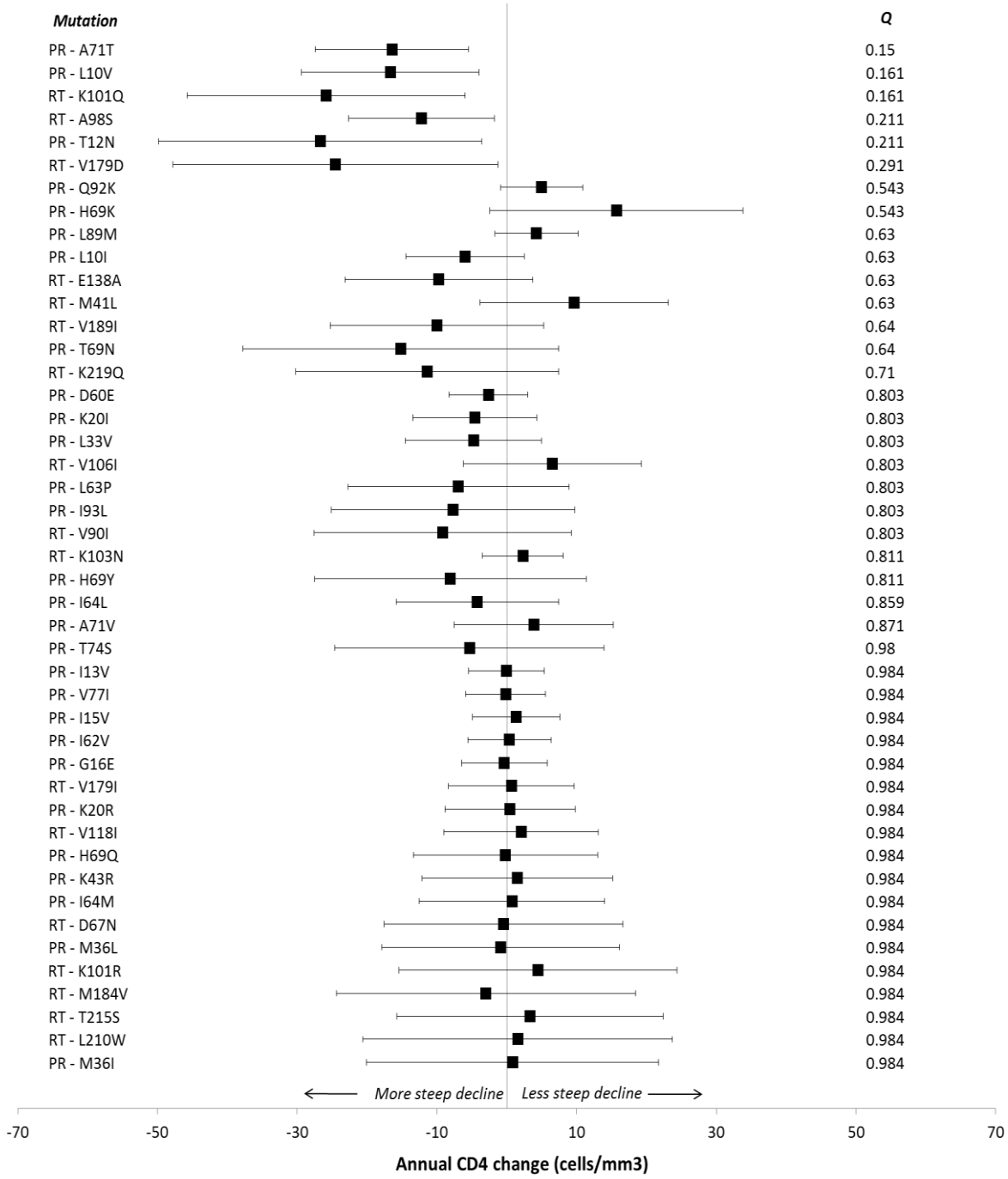
The first RT and PR clusters did not seem to have any marked effect on CD4 decline (p=0.37 and 0.17 respectively, Figure 7.10). In contrast, the second PR cluster, which contained minor mutations, was strongly associated with a less steep CD4 decline (p<0.001). The magnitude of the effect was not negligible; CD4 counts declined with 9 cells/mm^3 (95%CI=4-15) less per year among individuals whose mutation pattern was similar to that described by this cluster. The second RT cluster (including TAMs) was marginally associated with a slightly steeper CD4 decline (p=0.05).

Figure 7.7. The effect of any and class of transmitted (a) and treatment associated (b) resistance on CD4 decline



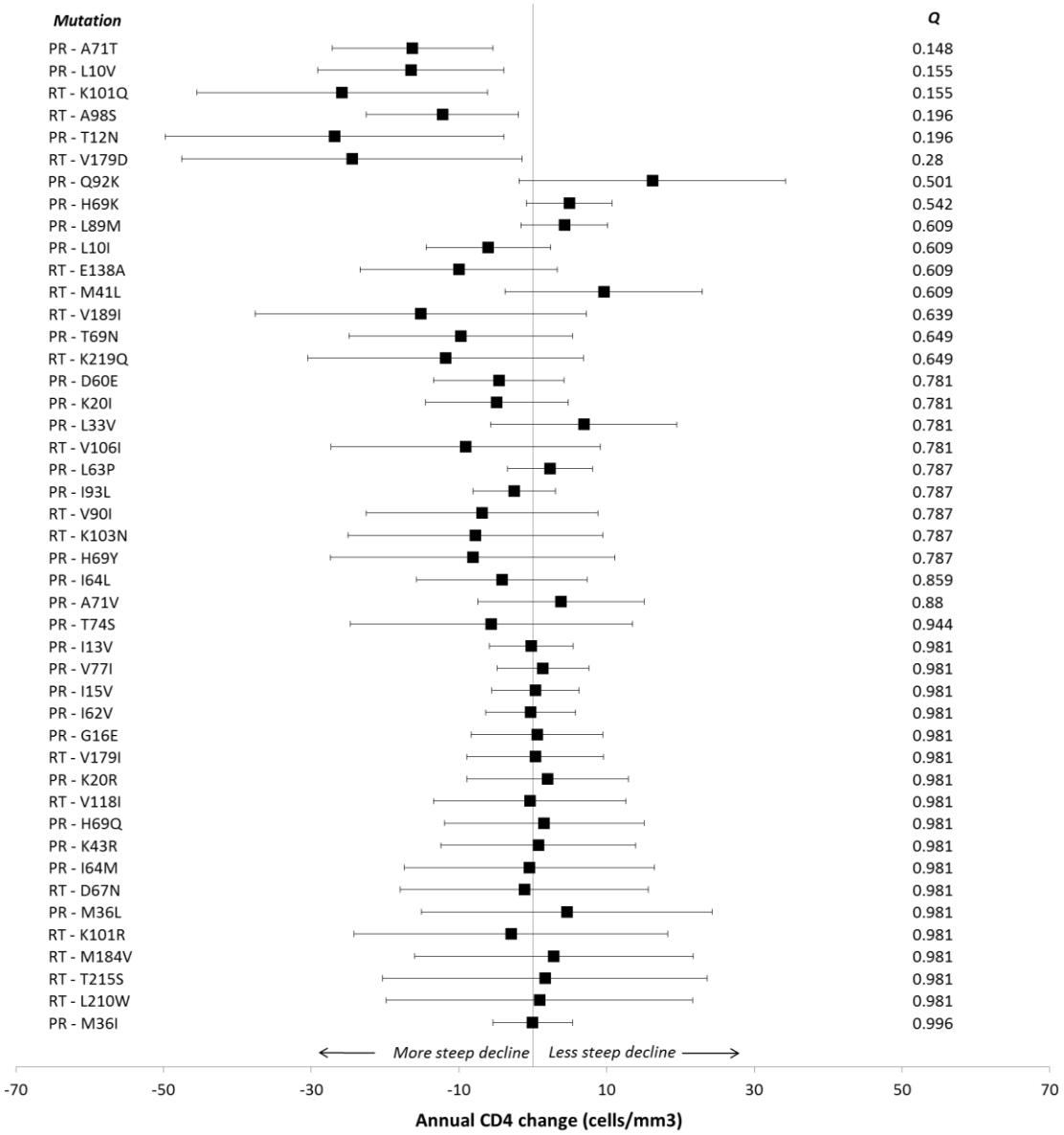
1. *Model 1 adjusted for risk group, subtype, calendar year and cohort. Model 2 additionally adjusted for viral load (log-scale, continuous).*
2. *322 individuals were excluded for missing risk group values*

Figure 7.8. Adjusted difference in CD4 decline according to the presence of specific mutations (Model 1)



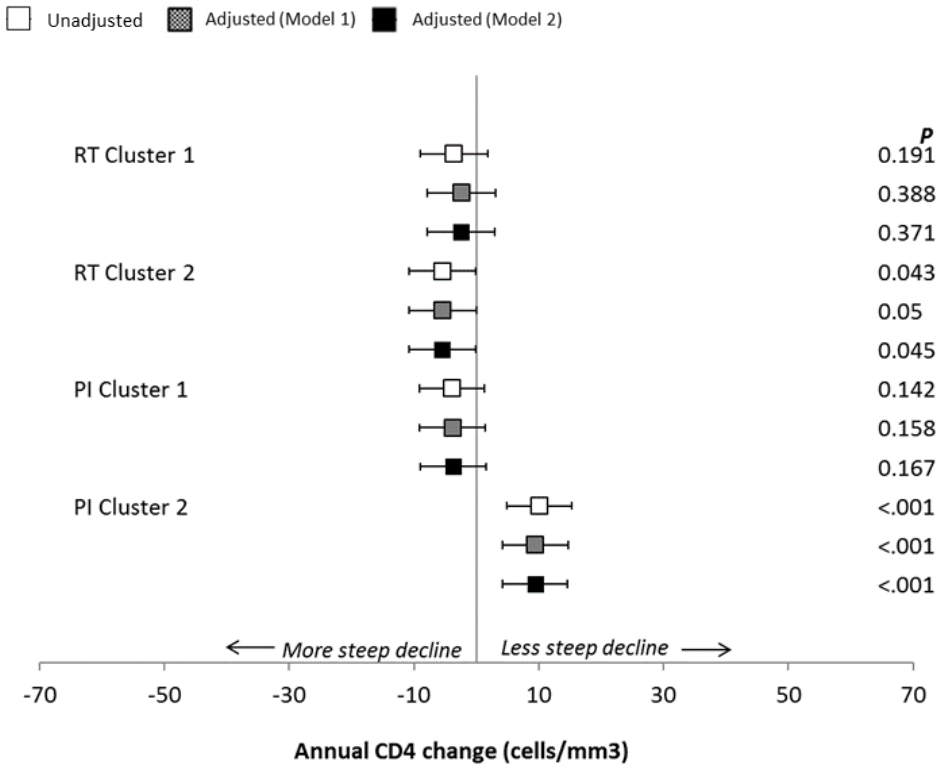
1. *Model 1 adjusted for risk group, subtype, calendar year and cohort.*
2. *322 individuals were excluded for missing risk group values*

Figure 7.9. Adjusted difference in CD4 decline according to the presence of specific mutations (Model 2)



1. *Model 2 adjusted for risk group, subtype, calendar year and cohort and the first VL measurement*
2. *322 individuals were excluded for missing risk group values*

Figure 7.10. The effect of clusters of mutations on CD4 decline



1. *Model 1 adjusted for risk group, subtype, calendar year and cohort. Model 2 additionally adjusted for viral load (log-scale, continuous).*
2. *322 individuals were excluded for missing risk group values*

7.4.5. Effect of resistance on VL set point

7.4.5.1. Associations between TDRM, DRM and VL set point

Associations between the detection of TDRM, DRM and the VL point can be seen in Figure 7.11. The estimated VL set point did not seem to vary according to the detection of TDRM after adjustment for the pre-specified confounders, including CD4 counts (difference=-0.05 $\log_{10}$ copies/ml, 95%CI=-0.12; -0.01, p=0.13). There was some weak evidence that the VL set point was slightly lower among individuals with NRTI and PI TDRM compared to those with no resistance (p=0.03 and p=0.04 respectively), but the magnitude of these differences was relatively small (difference=-0.08 $\log_{10}$ copies/mL, 95% CI=-0.16; -0.01 and -0.13 $\log_{10}$ copies/mL, 95%CI=-0.25; -0.01 respectively). There was no evidence that the detection of NNRTI TDRM was associated with the estimated VL set point (p=0.29). No associations were identified between any or class of DRM and the VL set point (Figure 7.11).

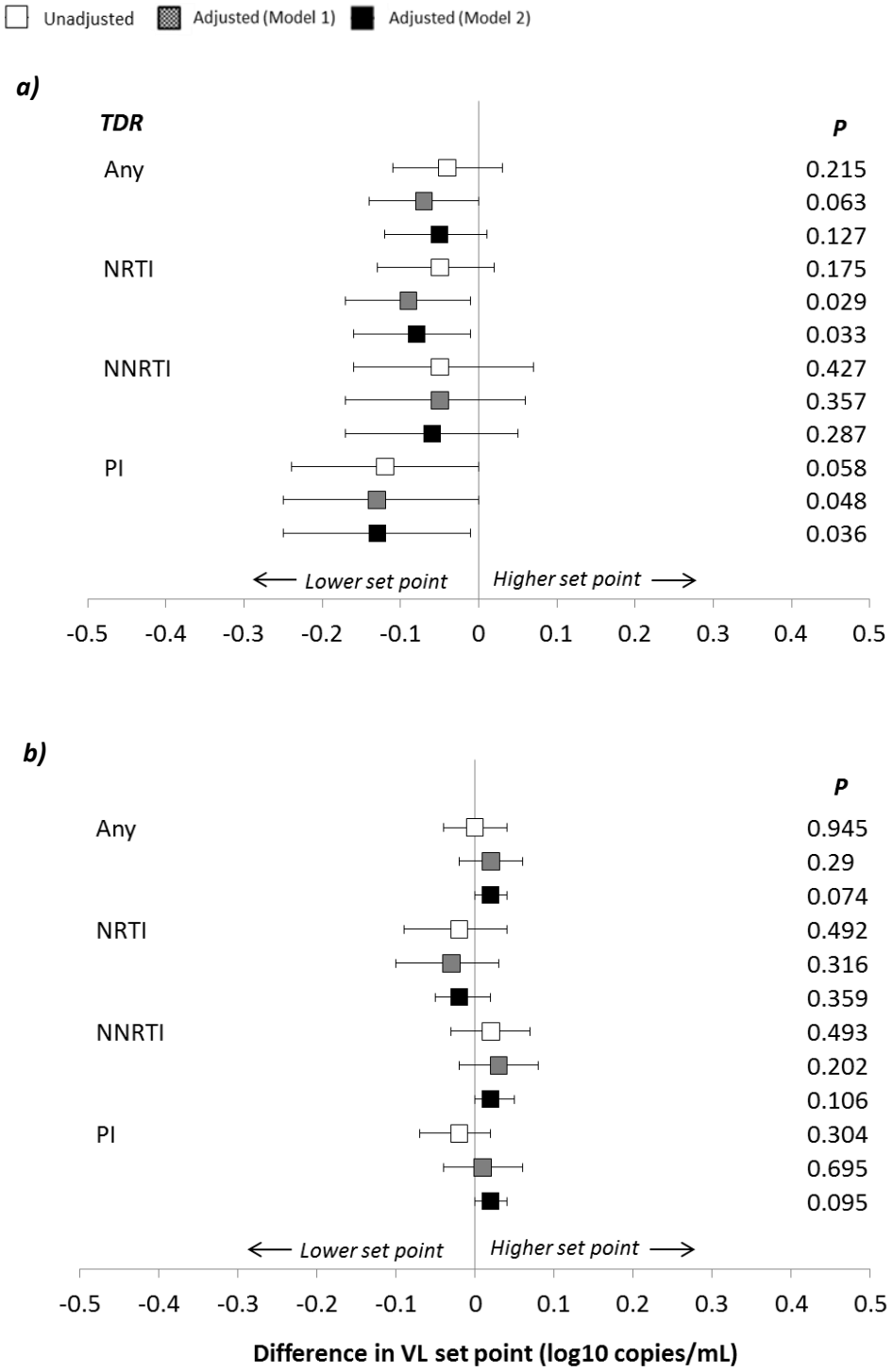
7.4.5.2. Associations between individual mutations and VL set point

After adjustment for all pre-specified confounders, there was evidence that the VL set point was lower among individuals who had either the G16E or the Q92K mutation in the PR (both q<0.001, Figure 7.12). The size of these differences was relatively small for G16E (-0.09, 95%CI=-0.13; -0.06 cells/mm^3/year), but larger for Q92K: -0.25, 95%CI=-0.33; -0.17 $\log_{10}$ copies/year. Adjustment for CD4 counts did not alter these estimates (Figure 7.13).

7.4.5.3.Associations between clusters of mutations and VL set point

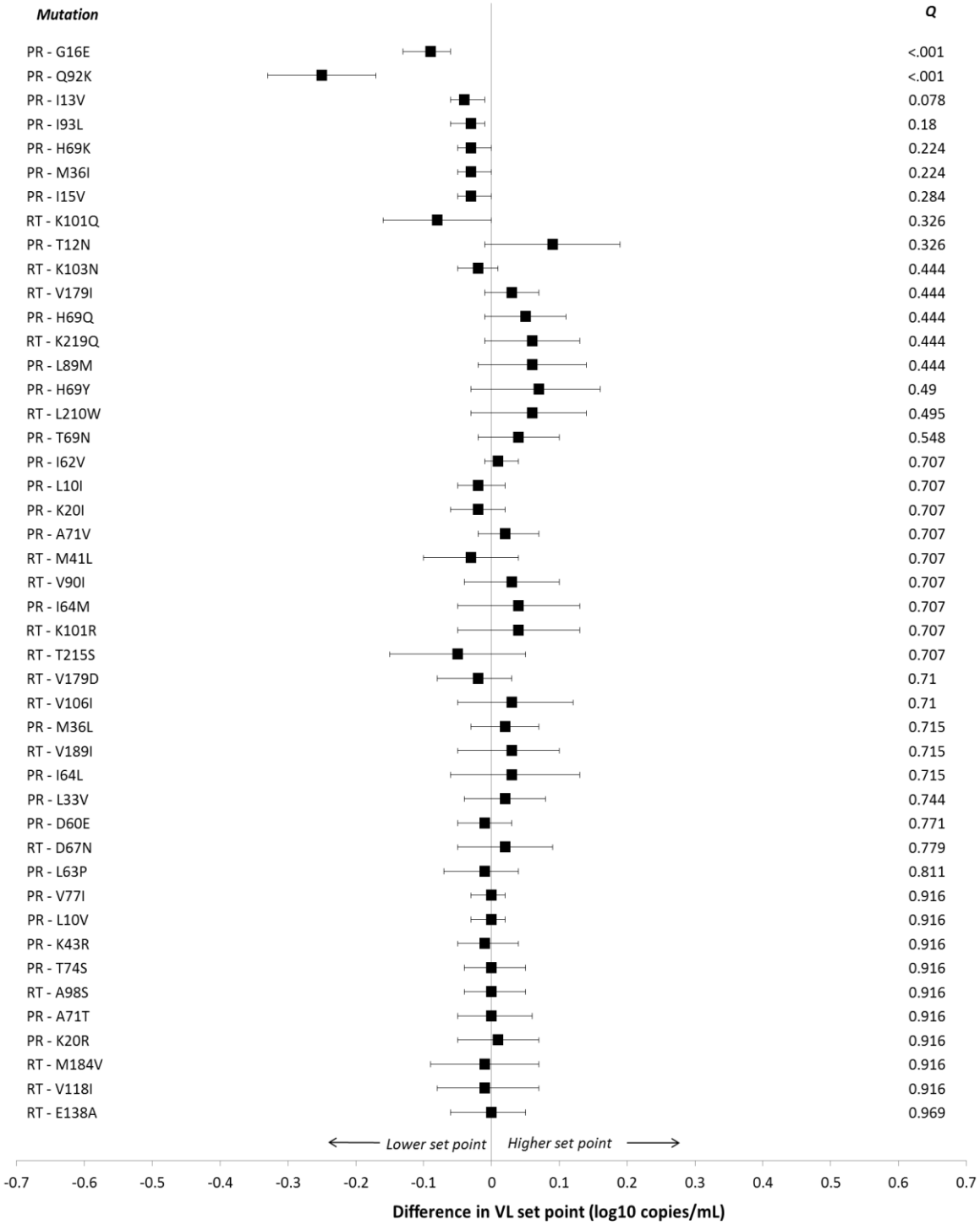
In agreement with the analysis of the impact of mutation clusters on CD4 decline, there was reasonable evidence suggesting that individuals whose mutation pattern aligned closely with the second PR cluster had lower VL set points both in univariable and multivariable analyses (adjusted p=0.004; Figure 7.14). Among those whose mutation pattern closely aligned with this cluster, the VL set point was on average 0.10, 95%CI=-0.17; -0.01 $\log_{10}$ copies/year lower compared to that among individuals with no resistance. In contrast, the second RT cluster was only weakly associated with a higher VL set point in univariable analyses, but this association did not persist after adjustment for confounders.

Figure 7.11. The effect of any and class of TDRM on estimated VL set point



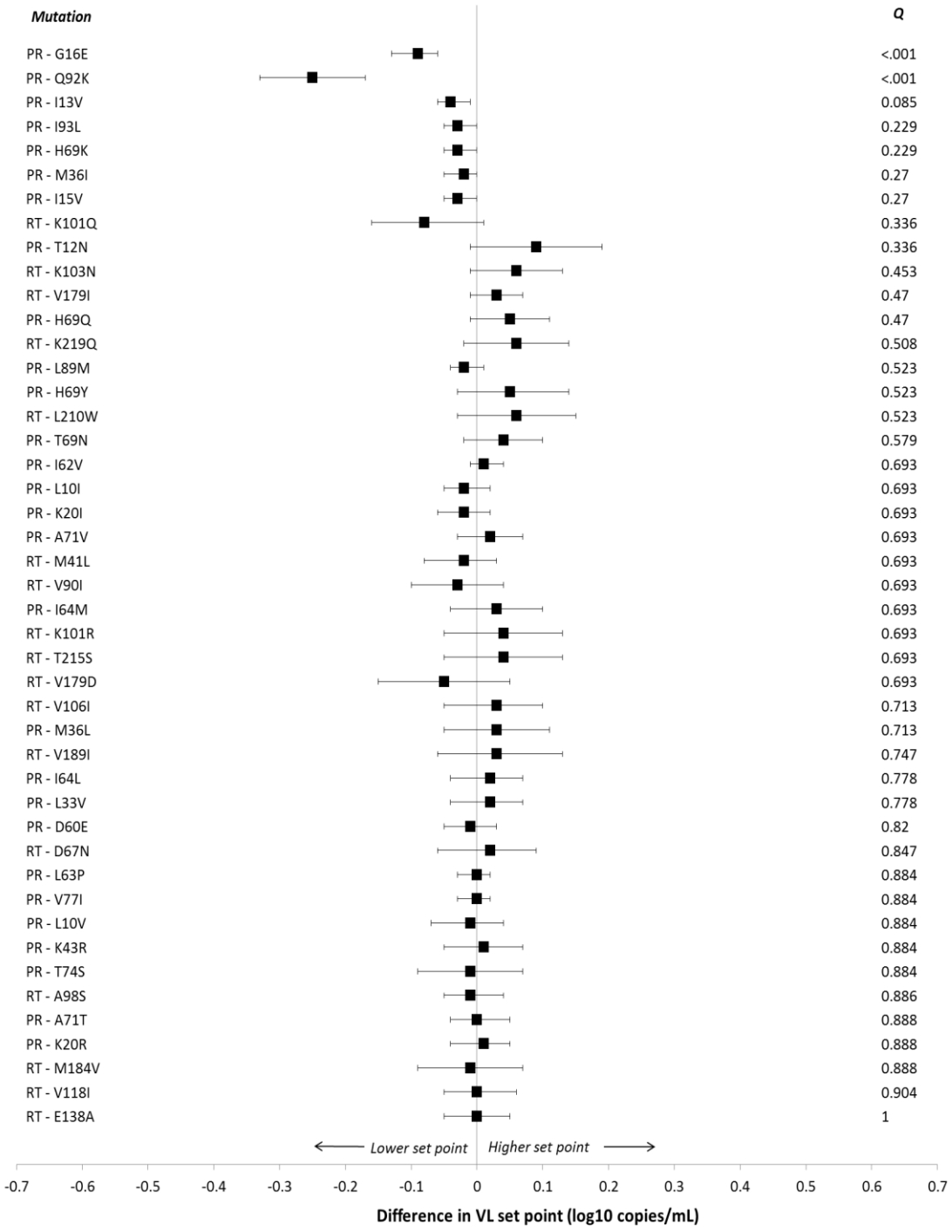
1. *Model 1 adjusted for risk group, subtype, calendar year and cohort. Model 2 additionally adjusted for CD4 counts.*
2. *322 individuals were excluded for missing risk group values*

Figure 7.12. Adjusted difference the VL set point according to the presence of specific mutations (Model 1)



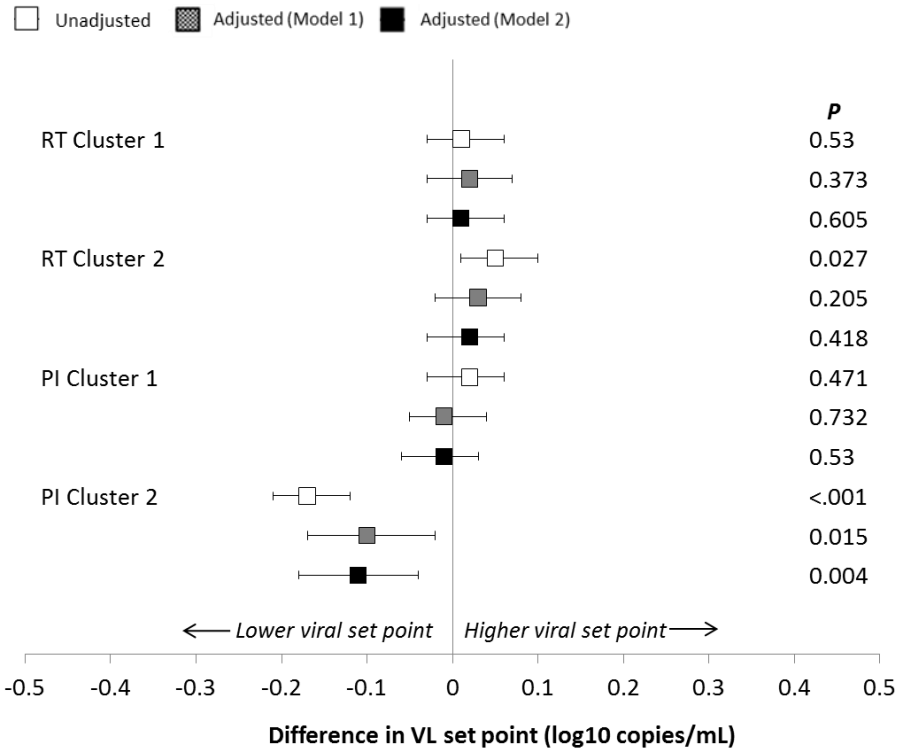
1. *Model 1 adjusted for risk group, subtype, calendar year and cohort.*
2. *322 individuals were excluded for missing risk group values*

Figure 7.13. Adjusted difference the VL set point according to the presence of specific mutations (Model 2)



1. *Model 1 adjusted for risk group, subtype, calendar year, cohort and CD4 counts.*
2. *322 individuals were excluded for missing risk group values*

Figure 7.14. The effect of clusters of mutations on the estimated VL set point



1. *Model 1 adjusted for risk group, subtype, calendar year and cohort. Model 2 additionally adjusted for CD4 counts.*
2. *322 individuals were excluded for missing risk group values*

7.4.6. Exploratory and sensitivity analyses

It can be hypothesised that HIV subtype might also affect CD4 count slopes and VL set point in the absence of therapy. A formal test for interaction between CD4 decline and subtype was borderline significant in multivariable models (p=0.04, difference= +7 cells/mm^3/year [2-13] comparing slopes among non-B to B subtypes), meaning that those infected with a non-B virus experienced slightly less steep CD4 declines. There was also strong evidence that subtype influenced the estimated VL set point (p<0.001), with those infected with a non-B virus having slightly lower VL set points (difference=-0.13 $\log_{10}$ copies/mL/year, 95%CI=-0.18; -0.08).

There were some notable differences in the results of the analyses evaluating the association between resistance and the CD4 outcome after restricting to individuals with a subtype B virus (Table 7.7). First of all, the effect of NNRTI DRM was stronger (adjusted difference=-10 cells/mm^3/year, 95%CI—17; -3, p=0.005). This latter finding appeared to be driving a weakly negative effect of any DRM on CD4 decline (adjusted difference=-6 cells/mm^3/year, 95%CI=-12;0, p=0.02). Tests for interaction indicated that the effect of NNRTI DRM varied significantly according to subtype (p=0.02). The effect of both the second RT and PR cluster on CD4 decline grew more extreme with wider confidence intervals, but interaction tests indicated that only the effect of the second PR cluster was likely to vary significantly according to subtype (p=0.007). Of note, only 3.6% of individuals with a subtype B virus reached the threshold to be classified as having a similar mutation pattern to that described by the second PR cluster, compared to 91.4% of individuals with a non-B virus. The evidence supporting an effect of the A71T and K101Q mutations on CD4 decline grew slightly stronger when restricting the analyses to individuals infected with a subtype B virus (both adjusted p=0.056), although interaction tests were only marginally significant (p=0.07 and 0.08 respectively).

There were also significant changes to the findings regarding the effect of individual DRM on the VL set point: no DRM were found to be associated with the VL set point when restricting the analysis to individuals with subtype B viruses. The point estimates for both the G16E and Q92K substitutions also moved towards zero when restricting to this patient group. Tests for interaction indicated that the effect of at least the G16E mutation differed significantly according to subtype (p=0.001 for G16E and p=0.11 for Q92K). There also appeared to be no effect of any or class of TDRM, DRM or clusters of mutations on the VL set point among individuals with subtype B viruses.

I also repeated the analysis after restricting to non-B subtypes, although this analysis was limited in terms of power, as only 1,471 (23.8%) individuals were infected with a non-B virus. Interestingly, the finding that the Q92K and G16E mutations may have a negative effect on the

VL set point grew stronger when restricted to non-B subtypes, as expected given the significant interaction tests. Although the effect of the second PR cluster on CD4 count decline was no longer significant when restricting to non-B subtypes, the point estimates were relatively similar in the restricted and main analysis (difference= 12 [6-31] cells/mm^3/year when restricted to non-B viruses, p=0.18) despite the evidence supporting an interaction. None of the other findings from the main analysis were significant in the non-B population (Table 7.8).

Removing EuroSIDA clinics from the analysis did not change any of the main conclusions, although the evidence that NNRTI DRM were associated with an increased CD4 decline grew slightly stronger (Table 7.9).

Table 7.7. Findings that were significant in the main analysis: Restricted to subtype B[1,2]

		Unadjusted		Adjusted (1)		Adjusted (2)	
		Difference (95% CI)	P	Difference (95% CI)	P	Difference (95% CI)	P
CD4 count slope							
DRM - NNRTI	Any	-10.97 (-17.81; -4.12)	0.002	-9.86 (-16.77; -2.95)	0.005	-9.84 (-16.68; -3.00)	0.005
RT FACTOR 2	Above Q3	-5.96 (-12.18; 0.26)	0.060	-6.19 (-12.48; 0.10)	0.054	-6.23 (-12.46; -0.01)	0.050
PR FACTOR 2	Above Q3	19.52 (4.92; 34.12)	0.009	19.78 (4.84; 34.72)	0.009	19.79 (5.01; 34.56)	0.009
VL set point							
TDRM - NRTI	Any	-0.05 (-0.14; 0.04)	0.254	-0.08 (-0.17; 0.01)	0.082	-0.07 (-0.16; 0.01)	0.089
TDRM - PI	Any	-0.09 (-0.23; 0.05)	0.211	-0.09 (-0.23; 0.06)	0.244	-0.12 (-0.26; 0.02)	0.083
PR FACTOR 2	Above Q3	-0.09 (-0.22; 0.03)	0.148	-0.09 (-0.21; 0.04)	0.184	-0.09 (-0.22; 0.03)	0.130
PR - Q92K	Compared to no	0.11 (-0.02; 0.23)	0.548	0.11 (-0.02; 0.23)	0.524	0.11 (-0.02; 0.23)	0.504
PR - G16E	Compared to no	-0.04 (-0.10; 0.01)	0.551	-0.04 (-0.10; 0.01)	0.524	-0.04 (-0.10; 0.01)	0.504

1. *Model 1 adjusted for risk group, subtype, calendar year and cohort. Model 2 additionally adjusted for VL/CD4 counts as relevant*
2. *322 individuals were excluded for missing risk group values*

Table 7.8. Findings that were significant in the main analysis: Restricted to non-B subtypes[1,2]

		Unadjusted		Adjusted (1)		Adjusted (2)	
		Difference (95% CI)	P	Difference (95% CI)	P	Difference (95% CI)	P
CD4 count slope							
DRM - NNRTI	Any	-2.30 (-15.65; 11.05)	0.736	-2.18 (-16.02; 11.67)	0.758	-2.17 (-15.94; 11.60)	0.757
RT FACTOR 2	Above Q3	-2.78 (-17.48; 11.91)	0.710	-2.74 (-18.38; 12.90)	0.732	-2.72 (-18.28; 12.84)	0.732
PR FACTOR 2	Above Q3	13.48 (-4.60; 31.56)	0.144	12.48 (-5.91; 30.87)	0.183	12.50 (-5.79; 30.79)	0.180
VL set point							
TDRM - NRTI	Any	-0.29 (-0.59; 0.01)	0.058	-0.17 (-0.48; 0.15)	0.305	-0.15 (-0.46; 0.16)	0.347
TDRM - PI	Any	-0.22 (-0.59; 0.14)	0.232	-0.16 (-0.54; 0.22)	0.412	-0.07 (-0.44; 0.30)	0.705
PR FACTOR 2	Above Q3	-0.11 (-0.27; 0.04)	0.156	-0.08 (-0.24; 0.08)	0.325	-0.09 (-0.24; 0.07)	0.281
PR - Q92K	Compared to no	-0.46 (-0.57; -0.34)	<.001	-0.47 (-0.58; -0.35)	<.001	-0.47 (-0.59; -0.35)	<.001
PR - G16E	Compared to no	-0.16 (-0.25; -0.08)	0.002	-0.17 (-0.26; -0.09)	0.002	-0.17 (-0.26; -0.09)	0.002

1. *Model 1 adjusted for risk group, subtype, calendar year and cohort. Model 2 additionally adjusted for VL/CD4 counts as relevant*
2. *322 individuals were excluded for missing risk group values*

Table 7.9. Findings that were significant in the main analysis: Removing EuroSIDA clinics

		Unadjusted		Adjusted (1)		Adjusted (2)	
		Difference (95% CI)	P	Difference (95% CI)	P	Difference (95% CI)	P
CD4 count slope							
DRM - NNRTI	Any	-8.31 (-14.88 - -1.74)	0.013	-8.31 (-14.88 - -1.74)	0.013	-7.23 (-13.89 - -0.57)	0.033
RT FACTOR 2	Above Q3	-5.64 (-11.92 - 0.64)	0.078	-5.49 (-11.95 - 0.97)	0.096	-5.53 (-11.93 - 0.88)	0.091
PR FACTOR 2	Above Q3	13.63 (7.54 - 19.72)	<.001	12.90 (6.58 - 19.21)	<.001	12.91 (6.65 - 19.17)	<.001
VL set point							
TDRM - NRTI	Any	-0.05 (-0.14 - 0.04)	0.261	-0.09 (-0.18 - 0.00)	0.056	-0.08 (-0.17 - 0.01)	0.070
TDRM - PI	Any	-0.15 (-0.29 - -0.01)	0.036	-0.16 (-0.31 - -0.02)	0.030	-0.16 (-0.30 - -0.02)	0.022
PR FACTOR 2	Above Q3	-0.16 (-0.21 - -0.12)	<.001	-0.08 (-0.16 - 0.01)	0.096	-0.09 (-0.18 - -0.01)	0.032
PR - Q92K	Compared to no	-0.27 (-0.35, -0.19)	<.001	-0.27 (-0.35, -0.19)	<.001	-0.27 (-0.35, -0.19)	<.001
PR - G16E	Compared to no	-0.11 (-0.15, -0.07)	<.001	-0.12 (-0.16, -0.08)	<.001	-0.12 (-0.16, -0.07)	<.001

1. *Model 1 adjusted for risk group, subtype, calendar year and cohort. Model 2 additionally adjusted for VL/CD4 counts as relevant*
2. *322 individuals were excluded for missing risk group values*

7.5. Discussion

7.5.1. Clusters of mutations

This analysis described the influence of HIV drug resistance, categorised in a number of different ways, on CD4 cell decline and the VL set point before the start of ART. I also used a simple data reduction technique, PCA, to study clusters of mutations. As expected given the absence of drug pressure, most of the clusters of mutations identified in this analysis differed slightly from those identified in individuals kept on failing treatment regimens in chapter 5. However, the second PR cluster was nearly identical to one identified in my prior analysis. This collection of minor PR mutations (involving positions 13, 20, 36, 69 and 89) did not have any effect on CD4 decline in the presence of failing treatment (Chapter 5). However, as shown in this analysis, individuals whose mutation pattern was close to that described by the cluster had slightly decreased CD4 declines before the start of ART and a lower VL set point. There was strong evidence that this effect varied according to subtype, and it appeared to be stronger among individuals infected with a subtype B virus.

Substitutions in position 36 are very common in non-B subtypes, and viruses carrying this substitution have a higher replication capacity than subtype B WT viruses (690,691). In other subtypes, such as G or CRF02-AG, substitutions in position 36 tend to appear with substitutions in position 20. It has been suggested that the combination of these mutations may present a selective advantage to the virus (692). With this respect, my findings are somewhat counterintuitive as mutations associated with a higher replicative capacity would be expected to cause a higher VL set point and faster CD4 count decline. However, the identified cluster also contained a number of other minor PR mutations. It is possible that the effect of the 20 and 36 mutations differ depending on the detection of other substitutions that form part of this cluster. On the other hand, one small previous study has found substitution 20I to be significantly correlated with lower VL during primary HIV infection (693), which provides some support for this cluster being associated with a less virulent form of HIV disease. However, it is important to note that I cannot rule out that unmeasured confounding or misclassification of the data affected the result. Only very few individuals among those infected with a subtype B virus had mutation patterns that closely resembled that described by the second PR cluster, and individuals infected with a non-B virus also had less steep CD4 declines and lower VL set points compared to those infected with a subtype B virus. It is possible that there is some misclassification of subtype in the dataset, and that this cluster is a marker for subtype. I also hypothesised that the presence of major mutations, not necessarily reaching the threshold for classification in the the PCA, may still be influencing the results. When I included “presence of

a major PI mutation" in the multivariable models as an indicator variable, the effect of the PCA cluster remained significant but grew weaker, and the point estimate moved towards zero (data not shown). This indicates that some of the effect may at least in part be mediated by major PR mutations.

7.5.2. Effect on CD4 count decline

Despite the large sample size, I was not able to find any definitive evidence that the detection of primary resistance mutations influenced CD4 decline. There was some weak evidence that NNRTI DRM were associated with a steeper CD4 decline, although this was not the case for NNRTI TDRM. The effect also appeared to be largely restricted to individuals infected with a subtype B virus, although the small sample size for the analysis restricted to non-B viral strains makes it hard to draw accurate conclusions. The modest fitness cost of most NNRTI mutations is relatively well described (507), and an increase in the speed of disease progression among individuals carrying NNRTI mutations would be biologically plausible. However, adjustment for VL did not shift the estimates markedly. Although this seems to suggest that any effect of these changes on the CD4 count slope acts through a mechanism other than higher viral replication and fitness, it is also possible that adjustment for the VL did not accurately estimate the direct total effect of resistance on CD4 decline (618) . This should, as outlined in Chapter 5, be borne in mind when interpreting the findings. There are a number of differences between the NNRTI TDRM and DRM lists that could explain why an association was found for NNRTI DRM but not TDRM. Of note, relatively strong but non-significant effects were found for two NNRTI DRM not included on the TDRM list: V179D and K101Q. K101Q is weakly selected among patients receiving NVP and EFV, whereas V179D is a polymorphic mutation that contributes to a lower susceptibility for all NNRTI's (394). Interestingly, individuals carrying the V179D mutation were found to have significantly steeper CD4 declines during failing treatment compared to those without the mutation in one of my previous analyses (Chapter 5). This mutation has previously been found to have high relative replicative fitness compared to other common NNRTI mutations when assessed in growth competition experiments (628).

No individual mutations were found to be associated with CD4 slopes, although patients infected with viruses harbouring A71T and 10V substitutions in PR region had non-significantly steeper CD4 declines compared to those with no resistance. Previous *in vitro* studies have linked changes in positions 10 and 71 with fitness recovery during treatment with protease inhibitors (694,695), and evidence from an *in vivo* study has suggested that neither the L10I/V nor the 71V/T mutation reduce the relative fitness of the virus once treatment is stopped (623). It is possible that these and other compensatory mutations occurring outside of the active site of the protease act to stabilize the structure of the enzyme, thereby restoring

fitness during the presence of selective drug pressure. Unlike major resistance mutations, these changes do not seem to impair the replicative capacity of the virus in absence of drug pressure, and could even confer an increased fitness compared to WT viruses which could manifest as a steeper reduction in the CD4 count before the start of ART. Theys et al have previously found a number of polymorphic mutations in the PR gene to be associated with a higher VL, lower CD4 count and higher estimated fitness from a fitness landscape (669). These mutations included A71T and L10V. Although this is intriguing, neither A71T nor L10V were associated with a higher viral set point in my analysis, as would be expected if they were associated with a markedly higher replicative capacity. It is also important to note that after correction for multiple testing I could not rule out that the effects found were due to chance.

Other authors investigating the relationship between resistance and CD4 counts before the start of ART have tended to study associations between any TDRM and CD4 counts at a single point in time. As shown in the literature review, the results have been conflicting (375,669,676–679,681,682). One of the largest studies describing longitudinal CD4 count changes was conducted by Pillay et al, using data from the CASCADE collaboration. They found evidence that CD4 counts declined faster among patients with TDRM, but only during the first year of infection (375). Unfortunately, the date of infection was not available in my dataset, and therefore the hypothesis that TDRM have a differential effect by varying time since the date of HIV seroconversion could not be directly evaluated.

7.5.3. Effect on the VL set point

I found weak evidence that the detection of transmitted NRTI and PI resistance was associated with lower VL set points, but the overall differences between individuals with and without resistance was small – around 0.1 $\log_{10}$ copies/mL – for both associations. It is unclear whether a difference of this size would have an impact on the transmission dynamics of HIV on a population level or on clinical progression as a result of a reduction in virulence. There were slightly larger differences between individuals who had the G16E and Q92K mutations and those who had no resistance, with both mutations being associated with lower VL set points. The finding appeared to be restricted to individuals infected with non-B viruses. G16E is a minor PR mutation included in the IAS list, whereas Q92K was selected for inclusion by the manual scanning of the Stanford HIVdb and is not included in any of the major resistance systems. I was not able to identify any studies which evaluated the potential impact of these mutations on the viral set point or the replicative capacity of viral strains more generally, and the mechanism by which they could affect virulence is not elucidated.

A number of authors have described the relationship between TDRM detection and VL values (669,678,681,682,686). Harrison et al analysed data from the UK CHIC cohort and the UK HDRD. Although they did not find any evidence that resistance to a single drug class was associated with VL in either univariable or multivariable analyses, they did find some evidence that the M184V mutation was associated with a lower baseline VL (686). In this analysis, I did not find any evidence that the M184V mutation had such an effect on either CD4 counts or the VL set point. It should be underlined, however, that resistance mutations that markedly impair HIV replicative capacity, such as M184V, are likely to wane over time in the absence of treatment due to overgrowth by more fit variants (380). The prevalence of M184V in this analysis was relatively low, and it is possible that I did not have sufficient power to detect any possible associations.

7.5.4. Differences according to subtype

The differences observed in CD4 count slope and set point according to subtype are interesting, and in agreement with findings from Keller et al (696), who found that individuals infected with a non-B virus experienced slower rates of disease progression. However, the "non-B" categorisation is relatively broad, and is likely to include subtypes that could both increase and decrease the speed of disease progression (697). Due to small numbers, it was not possible to conduct analyses stratified by specific non-B subtypes, such as D or A. A number of the main findings appeared to vary significantly according to the subtype of the virus, with the possible effect of NNRTI DRM being apparent largely among individuals infected with a subtype B virus, whereas the effect of individual PR mutations G16E and Q92K appeared to be primarily present among individuals infected with non-B viruses. It is not clear whether these variations in the effect estimates according to subtypes are due to biological differences or whether the primary explanation can be found in socio-demographic differences between groups infected with different subtypes. As data on ethnicity and socio-demographics was not collected in the EU-TDR collaboration, it was not possible to adjust for these factors.

7.5.5. Strengths and limitations

My analysis strengths include the large sample size, the transparent methods for mutation selection, the comprehensive evaluation of both types of resistance, individual mutations and clusters of mutations and finally and the longitudinal nature of the analysis. However, there are also a number of significant limitations. The primary weakness is the lack of an available date of infection. I attempted to estimate a date of infection using the shape of VL trajectories by trying to identify time periods in which a peak in VL resembling that observed around HIV seroconversion was experienced. However the number of individuals who experienced a significant VL increase of >2 $\log_{10}$ copies/mL followed by a decrease was too low (1.9% of the

total population) to allow for sensitivity analyses restricted to people in whom the date of seroconversion could be estimated. A second limitation is the lack of repeated resistance tests for the vast majority of individuals, which meant that I had to make assumptions regarding how long mutations persisted for. For simplicity, I assumed that resistance was present throughout FU. As the median FU in this study was just over 1 year and TDRM can persist for several years (698), this does not seem to be an unreasonable assumption. Nonetheless, repeated resistance tests would have allowed for more accurate modelling of resistance data in a time-updated manner. A third limitation is the number of statistical tests conducted. Although I corrected for multiple testing using recommend methods (445), I cannot rule out that some of these findings represent chance findings.

The generalisability of the study could also be limited, as individuals who received a resistance test before the start of treatment are likely to differ in a number of ways from individuals with TDRM who were not tested. As data on the source populations for the EU-TDR dataset were not available, it was not possible to assess the frequency of pre-ART genotypic testing. Nonetheless, it seems reasonable to assume that not everyone who is infected with a TDRM strain is tested for pre-ART resistance. EuroSIDA includes a number of centres in eastern Europe, where pre-ART resistance testing has been reported to be relatively uncommon, occurring in only 17% of cases (577). If those tested differ from those who were not tested in significant ways, it could mean that my results are only applicable to a subset of TDRM-infected individuals who receive tests in clinical care. The data quality of the EU-TDR collaboration reflects the quality of the data control and management procedures in each contributing centre, and no central data cleaning or quality controls were undertaken at the time of data submission. As in most observational studies, it is therefore possible that there are data entry errors or misclassification present in the data that could have influenced my results.

As confirmed in my analysis, the natural history of HIV may vary according to the subtype of the virus (696,697). Although exploratory analyses were performed separately for B and non-B subtypes, I was not able to study specific non-B subtypes. It is not clear to what extent my results will be applicable to different settings where other subtypes predominate. I was also not able to investigate the effect of TDRM on treatment response in those who eventually started ART, as viral loads after treatment initiation were not available within the EU-TDR collaboration. However, this question has been previously investigated in large European collaborations (373) as well as in EuroSIDA (699). Finally, the prevalence of TDRM, and in particular the prevalence of individual mutations, was relatively low. Despite the large dataset, my analysis may have suffered from a lack of power.

7.5.6. Conclusions

Bearing the limitations in mind, my results suggests that a number of genetic changes in the HIV genome may have a small but significant impact on the CD4 cell decline and VL set point before the start of ART. In particular, individuals infected with a subtype B virus may experience steeper CD4 declines if they have evidence of NNRTI-associated DRM's, whereas individuals infected with non-B viruses may have lower VL set points if they carry some minor PR mutations: G16E or Q92K. A cluster of minor PR mutations in position 13, 20, 36, 89 and 69 may influence both the speed of CD4 decline and the viral set point, particularly among individuals infected with a subtype B virus. However, the effect sizes were relatively small, and it is not known to what extent variation in the VL set point of this scale will influence the probability of transmission. It is also important to note that none of the resistance exposures investigated were associated with a higher VL set point, which could indicate an increased risk of transmission. My findings should therefore not affect the roll-out and expansion of ART coverage. The benefits of wide-spread ART usage are also likely to significantly outweigh any negative impacts it may have on epidemic virulence. Nonetheless, it may be of value to directly measure VL and CD4 values as part of TDRM prevalence surveys in order to monitor any potential changes in the virulence of HIV, irrespective of whether these are driven by expanding TDRM prevalence, ART coverage or different evolutionary pressures. To fully understand the impact of primary drug resistance on HIV virulence and disease progression, future studies should measure the date of seroconversion and, ideally, estimate the replicative capacity of strains directly in order to definitively assess the impact changes in the HIV genome has on virulence. More specific suggestions for further studies are discussed in Chapter 8.

7.5.7. Dissemination of findings

An early version of these findings was presented at the 2016 Conference of Opportunistic Infections and Retroviruses (abstract nr: 486, Poster No 16-966) and at the 2016 HIV Drug Resistance Workshop, both in Boston (Appendix X). A manuscript is in preparation.

Chapter 8 . Discussion and Conclusions

8.1. Summary of Thesis Findings

Since the development and widespread deployment of cART, HIV-related mortality and morbidity has declined markedly (173,700). The emergence of drug resistance, which hampered the use of early therapies (187,189,281), has become less of a clinical concern in high-income settings due to the wide variety of drugs available and the remarkable potency of modern combination treatments (515). However, resistance remains a threat to the success of HIV treatment due to its potential to jeopardise the use of standardised first line ART regimens in settings where genotypic resistance testing (GRT) is not widely available (422,584,701). As the number of people living with HIV who are on treatment in low income settings increases, the number of people requiring second and third line regimens is also expected to increase (701). Although this should not prevent the expansion of HIV treatment, it does have financial implications and warrants both extensive surveillance and continued research into epidemiological and biological aspects of HIV drug resistance. European HIV cohorts, many of which were established at the beginning of the HIV epidemic, represent valuable data resources due to the breadth of data collected and the length of follow-up time available. Although geographical representativeness is a limitation, data of HIV-infected individuals included in cohorts in Europe can nonetheless be used to answer questions relevant for policy in both high and low income settings. The research conducted as part of this PhD thesis aimed to address outstanding questions in the field of HIV drug resistance that the data sources available to me were particularly suited to answer. These included questions regarding the recent epidemiology of HIV drug resistance in Europe, as well as investigations into the consequences of maintenance therapy which is primarily used in low income settings today. This chapter summarises the main findings, discusses the overall implications and limitations of the research and presents suggestions for further research.

8.1.1. Chapter 3: Patterns of Resistance Testing and Detected Drug Resistance in Europe

Routinely collected data from observational cohorts are commonly used to estimate the prevalence of resistance in different settings, although many different methodologies are employed in order to construct an appropriate denominator (336,473,475,478,493). In Chapter 3, I described the frequency of use of genotypic resistance testing (GRT) following virological failure (VF) over time in Europe, and describe the prevalence of resistance in people who were tested and had a genotypic result available within the cohort. My analysis indicated that only around 1/3 of individuals experiencing VF received a resistance test within 12 months of the

date of experiencing VF, and that this proportion decreased after 2004. In a sensitivity analysis using stricter definitions of VF, the proportion receiving a test was higher but still below 50%. This seems to indicate that the use of GRT following VF on average across Europe is well below that recommended by clinical guidelines (601). However, clinical decisions are complicated by a number of different factors, and there are several possible reasons that could explain why the observed frequency of testing was lower than what is recommended in treatment guidelines. For example, perceived poor adherence is likely to discourage clinicians from requesting a GRT. My results indicated that individuals of non-white ethnicity and people who had acquired their HIV through injecting drugs were less likely to receive a resistance test, and both of these groups tend to have suboptimal adherence and issues regarding access to treatment (511,512). I also found that compared to individuals in Southern Europe , those in Eastern Europe were less likely to receive a GRT, whereas those in Northern and Central Western Europe were more likely to receive a GRT. As these results were adjusted for a number of different demographic and clinical factors, this indicates that either differences in clinical practice or in access to different technologies could cause disparities in the standard of care that HIV positive people receive in different regions of Europe. However, it is also possible that there are differences in the case mix of patients studied that are driving the discrepancies that I could not fully adjust for.

In contrast, the prevalence of resistance in people who were tested at failure was relatively high, at almost 80%. There was a suggestion from the analysis that this prevalence peaked in 2003-2004, and that this was lower in both earlier and later calendar years. However, the decline in resistance detection observed after 2004 was less marked compared to what has been described in previous European studies (336,478), possibly as a result of the different European regions included. Indeed, geographical region also had an impact on the odds of detecting resistance, with individuals receiving care in Northern and Central Eastern Europe less likely to have detected resistance as compared to those in Southern Europe. Although resistance was detected in the majority of the tests, the proportion of individuals both experiencing VF and, of those, who had a GRT, declined drastically over time. This suggests that of all individuals on cART, it is a small and decreasing proportion who experience VF with drug resistance.

Taken together, these findings suggest that clinicians in Europe may use GRT in a targeted way: whereby individuals more likely to have resistance are also those tested. The geographical differences identified have implications for public health policy, particularly for EU-countries where minimising inequalities in health care is a political priority (702). It also suggests that continued surveillance of acquired drug resistance prevalence is crucial, to ensure that gains

made in wealthier European countries (478,515) are replicated across the region. The fact that acquired resistance on ART is declining is re-assuring also in view of the fact that less circulating resistant strains at VF should lead to a decrease transmission of resistant HIV.

8.1.2. Chapter 4: Long-term Virological Outcomes and Resistance Patterns among Treatment Experienced Patients Receiving Raltegravir

Relatively recently, a new class of HIV drugs have been brought to the market. Integrase strand transfer inhibitors (INSTI) have been licensed for use in the EU since 2007, and their use has increased considerably since due to their high virological efficacy and favourable side-effect profile. Although the barrier of resistance to first generation INSTI is relatively low, the second generation INSTI dolutegravir is reported to have a high barrier to resistance (528). In 2015, 15% of individuals in EuroSIDA on cART and under FU were receiving raltegravir, the first licensed INSTI. Although the virological efficacy of RAL has been shown in numerous clinical trials (519,521,524), there are not many reports on long-term outcomes of RAL in routine clinic settings. In addition, resistance patterns following VF to RAL have not been well described outside of a clinical trial setting. In Chapter 4, I characterised long term virological responses to RAL and described resistance patterns following VF to RAL by conducting an analysis which involved retrospective genotyping of stored plasma samples which were collected around the time of VF. I found that by 7 years from starting RAL for the first time, approximately 10% of individuals starting RAL with a suppressed VL had experienced VF. This was higher among those who started RAL with a detectable VL. Although risk factors varied according to whether individuals started RAL with a suppressed or raised VL, those with more advanced HIV disease, as indicated by low current CD4 counts and lower CD4 nadirs, were more likely to experience VF in both groups.

Relatively few individuals experienced VF overall (n=262, 11%), and even fewer had an available stored plasma sample in the timeframe that I defined (from 3 months before to the end of the VF episode; n=33, 13%). The genotyping success was also relatively low (N=11, 34%), primarily as a consequence of the low VL threshold (50 copies/mL) used as the inclusion threshold for selecting the plasma samples. This limited the resistance aspect of the analysis to a description of resistance patterns observed. Despite the small numbers, the number of individuals genotyped is similar to that included in previous similar analyses (534,545,551,553,556,566), possibly reflecting the relative rarity of VF on RAL in clinical care. Among those with integrase resistance, I found evidence of at least partial resistance to another drug in the INSTI class, Dolutegravir (DTG) among 2 out of 4 people with integrase resistance, due to the presence of the N155H + E92Q mutation in one sample and the Q148H+G140S mutations in the other.

My analysis included the longest amount of FU of any analysis of RAL efficacy in routine clinical care published to date, but the estimates of virological efficacy presented here are nonetheless in line with those of other observational studies (546,549). DTG can show efficacy following RAL failure, but this is limited by the presence of resistance. The detection of INSTI resistance mutations which can compromise the response to DTG or other drugs in the INSTI class supports the use of GRT following RAL failure to determine which individuals are potential candidates for use of INSTI as part of salvage regimens.

8.1.3. Chapter 5: The Effect of Drug Resistance Mutations on CD4 Count Decline in HIV Positive Individuals Maintained on a Failing Treatment Regimen

Both Chapter 3 and Chapter 4 confirmed that experiencing VF with drug resistance is becoming increasingly rare, but constructing suppressive regimens can still be a challenge for individuals with very complex resistance patterns, or in low income settings, due to a combination of acquired resistance over sustained periods of virological failure and limited drug availability. When a fully suppressive regimen cannot be constructed, it may be necessary to keep individuals on suboptimal treatment for extended periods of time. It has been hypothesised that the use of drugs that preserve resistance patterns associated with a less fit virus in such a maintenance regimen may result in a clinical benefit for these patients, but the evidence supporting this assertion is weak and conflicting (598,599). Therefore, the aim of Chapter 5 was to describe the effect of acquired drug resistance mutations on CD4 decline among HIV infected individuals maintained on a virologically failing regimen. This analysis found that overall, individuals with resistance experienced significantly less steep CD4 declines compared to those without any detected resistance. This is in line with previous research finding that the absence of resistance despite ongoing viral replication may indicate issues with adherence to ART (417,664). Viral rebound without resistance can occur when there is an absence of drug pressure, as the wild type strain tends to outcompete resistant strains due to its fitness advantage. Among people in whom resistance was detected, my findings indicated that those with any NRTI mutation, or a number of individual mutations in the RT (e.g. M184V, D67N, T215Y) and PR region (e.g., V82A or I54V) also experienced less steep CD4 declines compared to those with other mutations. In contrast, those with any NNRTI resistance,the NNRTI mutation V179D or NRTI mutation L74V mutation experienced steeper CD4 declines. I also identified clusters of resistance using a fairly novel statistical approach in clinical HIV resistance research (a principal components analysis - PCA), and found that a cluster of resistance mutations conferring NNRTI resistance, including the K103N mutation, was associated with steeper CD4 declines. Conversely, a cluster of mutations conferring broad PI resistance was

associated with less steep CD4 declines. I also partially explored the hypothesis that the effect could be entirely mediated by viral load. Although results are not conclusive, it could not be ruled out that that there is a direct effect of the mutations on CD4 count decline, or that this effect is mediated through alternative pathways.

The mutations I found to be associated with less steep CD4 declines have been reported to confer reduced viral fitness, so my findings support the hypothesis that lower viral fitness could be clinically beneficial (507,703). If my findings are confirmed, it would suggest that the inclusion of LAM/3TC and a boosted PI is a good option for maintenance therapy, whereas maintaining an individual on an NNRTI based regimen for extended periods of time should be avoided. The results also provide support for expanding access to VL monitoring in low income settings, which would allow rapid detection of VF to first line, NNRTI-containing, cART. The findings from the PCA further support the avoidance of NNRTI agents as part of any salvage regimen, but also highlights that the effect of a single mutation on clinical outcome is likely to be complex to predict as it, at least in part, depends on other mutations.

8.1.4. Chapter 6: Rate of Accumulation of Drug Resistance Mutations During Virological Failure According to the Level of Viral Replication

One of the primary concerns when individuals living with HIV are maintained on a failing treatment regimen is the risk of further accumulation of resistance. In chapter 6 I estimated the rate of accumulation of drug resistance among people maintained on the same failing treatment regimens, and focused on describing how the level of viral replication might affect the rate of resistance development as this is not well described in the literature. I found that resistance accumulated at a rate of 1.51 (95%CI=1.37-1.68) new mutations per year. This is the equivalent of individuals, on average, accumulating approximately 1 new mutation per every 7 months and losing 1 drug option for every 24 months of being maintained on a failing regimen. Accumulation rates were highest for M184V (0.17, 95%CI=0.14-0.20 events/year), the K103N (0.07, 95%CI=0.05-0.09 events/year) and L90M (0.06, 95%CI=0.04-0.08 events/year) mutations. Although these estimates come from relatively early calendar years of HIV-infected patients seen for care in Europe , they are very similar to rates of resistance accumulation observed in other geographical settings (645,646).

Of note, the rate of resistance accumulation did not appear to vary according to VL values at the start of a VF episode nor the VL peak reached during the episode. However, VL measures that took into account VL changes throughout the episode: the average VL level, estimated VL slope and copy years viraemia (VCY) were all associated with the risk of resistance development. The risk of developing resistance appeared to be lowest when the average VL level during the episode was between 5,000-10,000 copies/mL or when the VL during the

episode increased with less than 0.12 log_{10} copies/mL/year or when it actually decreased. These results are consistent with the notion that there is faster selection of resistance when there is some, although suboptimal, drug pressure. Indeed, individuals with steeper VL increases of more than 0.21 log_{10} copies/mL/year or high VCY values over the failing episode were found to experience relatively low levels of resistance accumulation

One the aims of this analysis was to evaluate whether it was possible to identify a VL threshold close to the beginning of a VF episode below which resistance was unlikely to accumulate. As mentioned above, the risk of detection of resistance during the failure episode seemed not to correlate with the initial level of viral load and therefore my analysis failed to identify such a threshold. This suggests that clinically important resistance is still able to develop even at relatively low VL values, and supports the recommendation to use a relatively low threshold for defining VF (72). The lack of an association between peak VL, VL at the start of a VF episode and resistance development also suggests that VL measures taken at a single point in time are not strong predictors of the consequent risk of resistance development. Among patients who are maintained on a failing drug regimen, VL and CD4 should be frequently monitored so that issues with adherence can be identified as soon as possible and risk of development of resistance minimized. In low income settings, efforts should focus on expanding access to second and third line treatment regimens to allow for timely ART switches after first-line failure.

8.1.5. Chapter 7: The Effect of Primary Drug Resistance on CD4 cell Decline and Viral Load Set Point in HIV Positive Individuals Before the Start of ART

Maintaining individuals on a failing treatment regimen also has implications for the transmission of resistance. Transmitted drug resistance (TDR) has been associated with an increased risk of VF to first line regimens unless GRT is used before the start of ART to guide treatment (373), but it may also influence the speed of HIV disease progression in absence of ART (375,669). Any changes in pre-treatment CD4 counts/the viral load as a result of treatment associated or transmitted drug resistance mutations could indicate that the transmission dynamics of the HIV epidemic could change if the expansion in ART coverage is followed by an increase in the prevalence of transmitted drug resistance. To investigate part of this hypothesis I used data from the EU-TDR collaboration and EuroSIDA to quantify the impact of a number of different mutations on both the CD4 count decline in the absence of ART and the viral load set-point after infection.

I found little evidence for an association between transmitted resistance mutations, resistance mutations commonly classed as treatment associated and the speed at which CD4 counts

declined in absence of ART. There were weak associations between some classes of transmitted resistance (PI, NRTI) and the VL set point, although the absolute size of these effects was relatively small (a difference of approximately 0.1 $\log_{10}$ copies/mL when comparing people with or without mutations to these drug classes). Two individual mutations were associated with lower VL set points: the G16E and Q92K mutations. These are typically classified as minor PR mutations, and I was not able to identify any basic science studies investigating their effect on viral fitness. Of interest, although no individual mutations were significantly associated with CD4 decline after adjustment for multiple testing, there was some indication that two other polymorphic PR mutations that have previously been associated with fitness restoration in viral isolates, A71T and L10V, were associated with steeper CD4 declines before the start of ART (669,694,695).

As in Chapter 5, I studied the pattern of mutations using a PCA approach to identify clusters of mutations. A cluster of minor PR mutations involving substitutions at positions 20, 36, 69 and 89 was identified in both the analyses shown in Chapter 5 and Chapter 7. Pairwise correlations between mutations in position 20 and 36 have been previously reported (631,632), as have a clustering of mutations in position 20, 36 and 69 (630). These mutations appear particularly often among non-B viruses. Although the analysis in chapter 5 showed no evidence that this cluster affected CD4 decline in people receiving a virologically failing treatment, individuals whose mutation pattern closely aligned with the cluster had less steep CD4 decline and lower VL set points in absence of ART. It is not clear whether this mutational pattern represents a survival advantage to the virus, or if the clustering appears as a consequence of structural necessity among certain viral strains or subtypes (330,696). Regardless of the possible explanation, these data further support previous findings that patterns of mutations, particularly minor or polymorphic changes in the PR gene, are likely to have an important impact on pre-ART CD4 counts and VL (669). However, the effect that this may have on the transmission dynamics of HIV is not known. Future studies investigating this question should ideally use methods that take the entire genomic variation into account as well as utilising direct estimates of replicative fitness from laboratory studies. Further biochemical and structural analyses of the protease gene are also needed in order to explain why and how specific patterns of PI resistance emerge.

8.2. Limitations

Limitations specific to each of the five analyses were discussed in each chapter, but some limitations are of a more general nature and arose as a consequence of the observational nature of the data. These are discussed in more detail in the following paragraphs.

8.2.1. Data representativeness and generalisability

Most of the analyses in the thesis have been carried out using data from the EuroSIDA cohort. Although a strength of the EuroSIDA dataset is the representativeness achieved as a consequence of the pan-European data collection and minimal exclusion criteria applied as part of EuroSIDA recruitment, there are some limitations concerning generalizability. Firstly, patients attending EuroSIDA clinics may differ in important ways from those attending non-EuroSIDA clinics. EuroSIDA participating clinics tend to be university affiliated, larger centres of excellence based in urban areas. It is likely that the care provided at these centres is not representative of the care provided in an entire country or region. This is particularly an issue for the analyses in which resistance outcomes have been compared across geographical regions. Although limited numbers necessitated the grouping of clinics into regions, there is likely to be significant intra-regional variability in the quality of care provided (704). My results identify potential shortcomings in the clinical care provided in Eastern Europe, but it is not possible to establish whether this is true for the whole region or only for specific countries or clinics within a region.

Some of the questions addressed in this thesis might be of relevance to clinical care in low income settings. However, the composition of the HIV epidemic differs according to geographical regions, with different subtypes dominating in different regions (113). This is a clear limitation, and findings reported here should ideally be confirmed in cohorts conducted in the settings where any policies deriving from the findings are most likely to apply.

8.2.2. Data availability and missing data

Although using cohort data allowed me to access more detailed clinical information than those typically collected for in surveillance or registry databases, there was nonetheless limited information available on certain key variables. In particular, the lack of availability of a validated adherence measure in both the EuroSIDA and UK CHIC cohorts is a significant limitation for the majority of my analyses. For example, knowing a person's adherence status would have been necessary in order to validate the assumption that on-going viral replication without detected resistance is a consequence of very low adherence and an absence of selective pressure. However, although I was not able to investigate this directly, it has been found to be an accurate assumption in other studies (417,664). In addition, the lack of routine and reliable data on adherence to ART is a limitation shared by most European HIV cohorts.

Measuring adherence is complex, and although several different methods for estimating adherence have been developed there is no gold-standard measure employed across HIV cohorts (705). In general, direct methods, which involve measuring the amount of drug or drug metabolite directly, are considered more accurate than reported adherence, whether this is self-reported or reported by the treating clinician. As HIV cohorts evolve with the changing HIV research agenda, a simple, validated measure of adherence would represent a valuable additional data item. Such data would not only help answer some of the questions raised by my research, but also open up a variety of other research questions. For example, variation in adherence levels over time, in different countries and in relation to different type of drug regimens could be examined.

Missing data is also an issue particularly relevant to resistance data. As explained in Chapter 3, not every person who experience VF with resistance had a resistance test available in the database. This can be due to a number of reasons, some of which could be related to the outcomes of my analyses resulting in possible endogenous selection bias. However, although I took the approach of only describing resistance prevalence among those with an available test result, recognising the limitations of this approach, other strategies have been previously explored using EuroSIDA data (473).

8.2.3. Data quality

The data sources used here contain information from a large number of individuals followed for long periods of time. In EuroSIDA, quality control visits and accuracy checks are completed by the coordinating centre as outlined in Chapter 2, but it is likely that there are nonetheless errors present in the data used. Such errors can include data entry errors as well as issues that arise as a consequence of data management or processing. If these errors are systematic and differential, for example, more likely to occur among individuals from a particular geographical region, it can introduce bias in the analyses. The most effective way of ensuring high data quality is to prevent errors being introduced at the study design and data collection stage. Going forward, the EuroSIDA quality control procedures are being updated to reflect the challenges of a growing dataset. This has involved both streamlining data collection to focus only on key variables, but also introducing additional data control checks to be undertaken at the statistical centre.

8.2.4. Resource limitations

As with most research, the analyses presented here were constrained by finite resources. Genotyping, particularly using modern technologies, is still relatively expensive. This meant that I consciously applied relatively restrictive time-windows for the analysis in Chapter 4 when I sent samples for genotyping. The relatively low genotyping success rate in that analysis indicates that a cost-effective approach to further genotyping would prioritise samples with a higher VL, as this was the primary factor associated with genotyping success. Collaboration with industry or other funding providers may allow for more genotypic data to be collected going forward, and it is likely that the EuroSIDA resistance database will be expanded with more sequencing results from the integrase region. Future EuroSIDA publications might therefore be able to provide further insights into the risk of developing resistance on raltegravir.

8.2.5. Sample size and statistical power considerations

As no additional data collection was undertaken for most of the analyses in this thesis, the statistical power was fixed and determined by the existing data. Power was a particular issue in Chapter 5 and 7, where I aimed to establish the effect of individual mutations on CD4 count changes and the viral load set point. As the prevalence of single mutations can be low, and the magnitude of the detected association small, some of these estimates were relatively imprecise despite the large datasets used. Although the analysis in Chapter 5 and 7 are among the largest to date investigating the respective questions, it would nonetheless be of interest to expand existing collaborations to allow for even more precise estimates of these effects.

8.2.6. Unmeasured confounding

A problem in all observational studies is that of unmeasured confounding. Although multivariable statistical methods can account for bias introduced through measured confounding, it is not possible to adjust for confounders that were not measured. It is also possible that errors and inaccuracies in the measurements of certain variables result in residual confounding being present in the effect estimates, even after multivariable adjustment. I cannot rule out that the estimates presented here are affected by bias as the result of unmeasured or residual confounding. The only way to ensure that effect estimates are not affected by confounding is by conducting a large randomized clinical trial without any differential loss-to-follow-up. A trial could potentially be done to address the question set out in Chapter 5, regarding which regimens and ART drugs are most effective for use in maintenance therapy to preserve the CD4 count response. However, in order to recruit sufficient numbers of individuals that have no other treatment options available, the trial would need to involve a large number of centres and to enrol from sites mostly located in low income settings. The complicated ethical issues involved in conducting such a trial, where

people are maintained on an ineffective treatment when effective treatment options are available in other settings, makes it less likely that one will be carried out.

8.3. Clinical Implications and Further Research

Bearing these limitations in mind, my research nonetheless has a number of important implications. Firstly, the analyses in Chapter 3, and to a lesser extent Chapter 4, confirmed that aspects of clinical care for HIV vary significantly according to geographical region. Taken together with the findings from other analyses (575–577,579,706), this adds to a growing body of evidence showing that care for HIV in the Eastern European region lags behind that in other areas of Europe. Further research identifying the specific factors that explain these discrepancies, including on a health-systems level, would help design targeted interventions. Given that infectious diseases do not respect borders, a comprehensive and collaborative approach including a sharing of resources may be needed by countries in the WHO European region in order for the 90-90-90 target to be achieved.

The results shown in Chapter 3 also confirmed that experiencing VF with resistance is becoming increasingly rare. This confirms the well-known success of the introduction of modern cART (515). However, the results presented here also indicate that the use of GRT to detect resistance following VF is lower than what might have been expected according to current guidelines. Although this discrepancy between guidelines and clinical practice can be explained by actions that are consistent with good clinical practice and may not necessarily jeopardise clinical outcomes, it is important to further understand what motivates clinicians in different countries to request GRT for some patients and not others. Mixed methods studies including interviews and qualitative analyses might be able to address this question. Although the use of GRT has been shown to be beneficial in clinical trials conducted in earlier calendar years, no trials evaluating the role of GRT have been done following the introduction of more modern ART classes, such as integrase inhibitors. The cost-effectiveness of resistance testing following the introduction of NGS has also been questioned; although recent evidence suggests that the use of NGS is cost-saving compared to Sanger sequencing (707). As many European health systems are attempting to identify areas for cost-saving, future research should include further evaluations of the cost-effectiveness of resistance testing, using both NGS and Sanger sequencing, in the context of modern cART.

The research presented in Chapter 5 has some potentially important clinical implications. If the findings are replicated in other studies, including those in low income settings, it would suggest that current salvage regimens may benefit from the inclusion of particular drugs, such as Lamivudine and a boosted PI, whereas the use of NNRTI-containing regimens should be avoided. The findings also highlight the importance of expanding routine VL monitoring in low income settings so that those experiencing failure on first line treatment can be rapidly

switched from NNRTI-containing regimens. However, as mentioned previously, the ideal evidence to support the use of particular treatment and treatment switch strategies should come from randomized controlled trials. Although a trial evaluating different salvage regimens in low income settings would be complex to design, it could potentially provide very important information for clinicians faced with difficult treatment choices. In the absence of firm evidence from RCT's, it is important to focus efforts and resources on expanding treatment options in low income settings. The analyses from Chapter 5 would benefit from being expanded to include an investigation of the impact of integrase resistance on CD4 decline. This is particularly important given the recent consideration given by the WHO to potentially swap current first line treatments to DTG based regimens.

The potential benefit of maintaining a patient on a particular treatment regimen needs to be balanced with the risk of developing further resistance, which could contribute to the spread of transmitted resistance and jeopardise the use of newer ART drugs when these become available. The results shown in Chapter 6 indicates that new resistance is likely to develop at a rate of one new mutation for every 7 months of ongoing VF. Ideally, estimates of the rate of resistance accumulation would be calculated in studies where resistance testing is frequently repeated at set time-points for each individual. This would allow for a more accurate determination of the follow-up time contributed. An absence of sparse or missing genotypic testing results would also open up the possibility to perform extra analyses such as the use of marginal structural modelling to estimate the best time at which to switch ART to reduce the risk of resistance accumulation, or the use of standard regression models to accurately identify people who are most likely to develop further resistance so that they could be prioritised for treatment. Tools along these lines are already in development (643), and the research presented here supports the use of repeated VL measurements over the failure episode (rather than the level of copies/mL observed at the time of first failure for example) to identify those most likely to benefit from a treatment switch.

The analyses presented in Chapter 7 indicates that the detection of viruses with genetic changes in absence of ART is unlikely to greatly affect the viral set point or speed of CD4 decline among those who become infected with resistant HIV. However, the lack of a date of seroconversion complicates the interpretation of my findings, as it is impossible to establish exactly for how long the detected mutations have been circulating as dominant species. Further research investigating this question should ideally be carried out in cohorts where estimates of the date of seroconversion is available. A number of relevant analyses have been done in the CASCADE cohort, although these suffered from a relatively small sample size which limited their ability to investigate individual mutations (684). Alternatively, it could be valuable

to develop statistical methods that would allow for an estimation of the date of seroconversion on the basis of VL trajectories. The frequency of viral load measurements in the datasets used here was insufficient to fulfil this objective.

In general, the lack of certain key data in this thesis, such as adherence measures, dates of seroconversion and irregular and missing genotypic testing data suggests that future studies should consider expanding data collection in order to capture a broader set of variables than that provided by routine health care data. EuroSIDA is currently undergoing a restructuring in order to better respond to future research priorities, and this represents an opportunity to expand or amend the data collection. In addition to HIV resistance data, there will likely be interest in resistance to the new direct acting antivirals (DAA) used to treat HCV, as it seems to be causing failure in a not negligible proportion of HCV-infected individuals. An expansive and comprehensive plasma sample repository, as that held by EuroSIDA, represents an ideal resource to investigate many of these questions as additional data, including both resistance genotyping and potentially drug concentration measurements as a marker of adherence, can be generated retrospectively when resources are identified.

8.4. Concluding Remarks

HIV drug resistance has, fortunately, evolved from being a major barrier to successful treatment to, following the advent of modern cART, being of less clinical and of population level health importance. However, the theoretical potential for specific mutations to emerge and cause wide-spread impairment to the use of standard first line regimens highlights the importance of continued surveillance and research into this area. This thesis contributes both epidemiological data on HIV resistance testing, prevalence and incidence, as well as data that enhances our understanding of how drug resistance influences disease progression in the presence and absence of therapy. It is hoped that these findings can be used to both improve aspects of clinical care for people living with HIV, as well as to further the research agenda in the field of HIV drug resistance. Together with continued activism and advocacy for greater access to laboratory technologies and treatment, it is hoped that this can ensure greater health equity and a better quality of life for people living with HIV.

Appendix I: The EuroSIDA Study Group

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Appendix IV: The EU-TDR collaborating cohorts

The European Transmitted Drug Resistance collaboration (EU-TDR) includes the following cohorts/collaborators: Virolab project (Sloot PM, Boucher C, van de Vijer D, Vandamme A, Libin P, Theys K, De Luca A, Colafigli M, Di Giambenedetto S, Torti C, Quiros-Roldan E, Lapadula G, Ruiz L, Muller V, O Nualláin B), EuResist (Scientific Board: Bobkova M, Camacho R, Clotet B, Incardona F, Kaiser R, Karasi JC, Lengauer T, Mugusi F, Sayan M, Schmit JC, Rosen-Zvi M, Sonnerborg A, Vandamme A, Zazzi M)

Appendix V: Extra Mutations for Chapter 7

Selected manually from the Stanford Drug Resistance Database	Polymorphisms considered in Theys et al (2012)
pr92K	pr13V
pr12N	pr60E
pr33I	pr60E
pr69R	pr2V
pr12K	pr71T
pr72L	pr71V
pr61D	pr77I
pr55R	pr93L
pr20M	
pr36V	
pr12E	
pr17D	
pr18H	
pr75I	
pr11I	
pr37Q	
pr92R	
pr93M	
pr72R	
pr79D	
pr35G	
pr66V	
pr20V	
pr43I	
pr72K	
pr10R	
pr71I	
pr89F	
pr11L	
pr22V	
pr34D	
pr55N	
pr91A	
pr34K	
pr45Q	
pr66L	
pr67F	
pr10Y	
pr20L	
pr34Q	

Appendix VI: Publication arising from Chapter 3

HIV resistance testing and detected drug resistance in Europe

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Objectives: To describe regional differences and trends in resistance testing among individuals experiencing virological failure and the prevalence of detected resistance among those individuals who had a genotypic resistance test done following virological failure.

Design: Multinational cohort study.

Methods: Individuals in EuroSIDA with virological failure (>1 RNA measurement >500 on ART after >6 months on ART) after 1997 were included. Adjusted odds ratios (aORs) for resistance testing following virological failure and aORs for the detection of resistance among those who had a test were calculated using logistic regression with generalized estimating equations.

Results: Compared to 74.2% of ART-experienced individuals in 1997, only 5.1% showed evidence of virological failure in 2012. The odds of resistance testing declined after 2004 (global $P < 0.001$). Resistance was detected in 77.9% of the tests, NRTI resistance being most common (70.3%), followed by NNRTI (51.6%) and protease inhibitor (46.1%) resistance. The odds of detecting resistance were lower in tests done in 1997–1998, 1999–2000 and 2009–2010, compared to those carried out in 2003–2004 (global $P < 0.001$). Resistance testing was less common in Eastern Europe [aOR 0.72, 95% confidence interval (CI) 0.55–0.94] compared to Southern Europe, whereas the detection of resistance given that a test was done was less common in Northern (aOR 0.29, 95% CI 0.21–0.39) and Central Eastern (aOR 0.47, 95% CI 0.29–0.76) Europe, compared to Southern Europe.

Conclusions: Despite a concurrent decline in virological failure and testing, drug resistance was commonly detected. This suggests a selective approach to resistance testing. The regional differences identified indicate that policy aiming to minimize the emergence of resistance is of particular relevance in some European regions, notably in the countries in Eastern Europe.

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Keywords: antiviral drug resistance, Europe, HIV, logistic models, prevalence

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Introduction

Combination antiretroviral therapy (cART) has brought considerable clinical and public health benefits, by suppressing HIV-1 replication and consequently allowing $CD4^+$ cell counts to increase [1–3]. However, in cases of incomplete viral suppression, resistance to antiviral drugs may develop [4]. Such acquired drug resistance limits the number of available treatment options, compromises the benefits of cART by impairing the response to therapy and could also contribute to the transmission of drug-resistant HIV strains [5–7]. Given these clinical consequences, monitoring trends in resistance prevalence is important.

Prevalence estimates from observational studies tend to rely on resistance tests done as part of routine clinical care in order to access genotypic data. Whether or not a resistance test is performed following virological failure is a matter of clinical judgment and cost, but there are several clinical guidelines available to guide decisions. Current European as well as US guidelines recommend testing for drug resistance at both initiation of therapy and following virological failure [8,9]; however, this has not always been the case. Since resistance testing first became widely available in the late 1990s, clinical guidelines have changed. This is in part to reflect the results from trials showing that knowledge of a patient's resistance profile results in treatment benefits [10–13], and partly due to the implementation of technologies that allow genotyping at lower viral loads [14,15]. This, coupled with the fact that resistance testing technologies have become more widely available and affordable, has resulted in a changing proportion of ART-experienced individuals receiving resistance tests in clinics over time [9]. Such changes complicate estimates of resistance prevalence and in particular the comparison of prevalence estimates between different studies, as the denominator varies with time and between different studies and countries. This makes trends in resistance testing important for interpreting prevalence estimates, particularly when these are derived from observational studies.

For this reason, the aims of the current analysis were first, to describe regional differences and trends in resistance testing among individuals experiencing virological failure, and second, to describe regional differences and trends in the prevalence of detected resistance among those individuals who had a genotypic resistance test.

Study population and methods

The EuroSIDA study

EuroSIDA is a large, ongoing prospective cohort study of more than 18 000 individuals living with HIV. At the time of analysis, the study collected data from 111 hospitals in 34 different countries across Europe, as well as Israel and Argentina [1]. For this analysis, these countries have been grouped into five different regions (Southern Europe, Central Western Europe, Northern Europe, Central Eastern Europe and Eastern Europe, as detailed in Supplementary Digital Content 2, http://links.lww.com/QAD/A700) as described previously [16]. Recruitment started in 1994, and data were collected 6-monthly on standardized case report forms (CRFs). Variables collected include demographic information, $CD4^+$ cell counts, viral load measurements, as well as start and stop dates for all antiretroviral drugs used. All patients gave informed consent at enrolment, as described at www.cphiv.dk. An extensive programme of quality control is in place, details of which have been previously published [16,17].

Resistance data

Virological reports of resistance tests done by clinicians as part of routine clinical care are submitted to a centralized resistance database held at the IrsiCaixa Foundation, Badalona, Spain. The methods used to test for resistance differs depending on the centre, and our database contains very limited information on this. Clinicians can also indicate whether or not an individual's virus has been genotyped since the last clinic visit in the main CRF. These data give no genotypic information, and could therefore not be used to address the second aim of this particular study.

Resistance mutations were defined using the International Antiviral Society (IAS)-USA (2013) guidelines [18]. Throughout the article, 'any resistance mutation' will refer to at least one detected IAS-USA resistance mutation to nucleoside reverse-transcriptase inhibitor (NRTI), nonnucleoside reverse-transcriptase inhibitor (NNRTI) or protease inhibitor (PI) drug classes, excluding minor PI mutations. As the analysis focused on detected drug resistance at a single time point, resistance mutations were not carried forward in a cumulative manner.

Inclusion criteria

All individuals aged above 16 years with evidence of virological failure (having a viral load measurement >500 copies/ml while on ART after at least 6 months of ART exposure [8]) after 1 January 1997 were considered eligible for a resistance test and were included in this analysis. We consciously used a broad definition of virological failure for our primary analysis in order to maximize the number of individuals that we could include. For any given year, a person was included if they experienced virological failure in that year, and considered as having a resistance test associated with the virological failure in that year if they had a resistance test no more than 1 month before or 12 months after the date of virological failure. If the same resistance test could be linked to more than one virological failure date in different calendar-years, it was attributed to the virological failure date occurring closest to the test. Individuals contributed one measurement for each year in which they experienced

virological failure. This means that individuals could contribute data for more than 1 calendar-year, and were not excluded after they had their first virological failure or resistance test. The first included date of virological failure for each individual was considered as the baseline date for that person, and the characteristics of the study population at baseline were summarized.

Statistical methods

The proportion of individuals with a resistance test following virological failure (first aim of the study) and detected resistance after having a test (second aim of the study) was plotted against calendar-year with 95% confidence intervals (CIs). Logistic regression models with generalized estimating equations (GEEs) [19,20] were used to identify predictors and to test for changes in the prevalence of resistance testing and detected resistance. The rationale for which covariates to include in the multivariable models was based on clinical knowledge and previous publications [21,22], and included factors hypothesized to be associated with our exposures of interest and the relevant outcome. Adjustments are detailed in the relevant tables (Table 2, Supplementary Digital Content 3, http://links.lww.com/QAD/A700).

We also studied factors associated with virological failure in order to add to the context of the current analysis. This model was adjusted for age, sex, ethnicity, mode of HIV transmission, region, previous history of mono/dual therapy use, number of available previous resistance tests, $CD4^+$ cell count, previous history of virological failure and calendar-year.

P values of less than 0.05 were taken to indicate statistical significance. All analyses were conducted using SAS 9.3 (Statistical Analysis Software, Cary, North Carolina, USA).

Sensitivity analyses

Several sensitivity analyses (Supplementary Digital Content 5, http://links.lww.com/QAD/A700) were done using varying definitions of virological failure.

Results

Characteristics of the individuals showing evidence of virological failure

In total, 8469 individuals – 57.3% of all ART-experienced individuals with at least one RNA measurement – met our main definition of virological failure on at least one occasion and were included in the analysis (Supplementary Digital Content 1, http://links.lww.com/QAD/A700). The odds of experiencing virological failure declined over calendar time [adjusted odds ratio (aOR) 0.79 per more recent calendar-year, 95% CI 0.78–0.79, $P<.001$]. Compared to 74.19% (95% CI 72.9–75.4) of individuals in 1997, only 5.11% (95% CI 3.22–8.00) showed evidence of virological failure in 2012. There was evidence that this decline differed significantly according to region (interaction P value < 0.001). The odds of experiencing virological failure declined most steeply in Central Eastern Europe (aOR 0.90, 95% CI 0.87–0.93, $P < 0.001$). In contrast, the decline in the odds of experiencing virological failure was less marked in Eastern Europe (aOR 0.95, 95% CI 0.91–0.99, $P = 0.01$; Supplementary Digital Content 3, http://links.lww.com/QAD/A700). The lowest levels of virological failure in 2012 were seen in Northern Europe (3%), and the highest levels in Eastern Europe (15.1%; Supplementary Digital Content 3, http://links.lww.com/QAD/A700). The median viral load at failure varied with calendar-year, and decreased somewhat from 5831 (1500–30000) copies/ml in 1997/1998 to 4378 (1230–21902) copies/ml in 2011/2012 ($P < 0.001$). Conversely, the number of viral load measurements per patient per year decreased over time, from a median of 4 (3–6)/year in 1997/1998 to 3 (2–4)/year in 2011/2012 ($P < 0.001$).

The characteristics of the individuals showing evidence of virological failure at date of the first virological failure are shown in Table 1; the majority of the individuals included were men (75%) and white (86%), and 40% had acquired their HIV infection through sex with another man.

Resistance testing

Trends in proportion of individuals with a resistance test over time

Of the 8469 individuals experiencing virological failure, a total of 2676 (31.6%) were tested for resistance in at least one of the years in which they had evidence of virological failure. Among those who had a test, the median time between the date of virological failure and a resistance test was 0.7 months [inter-quartile range (IQR) 0–4 months; range 1–12 months]. Of those with at least one resistance test, 60.7% had one test, 23.5% had two tests, 8.5% had three tests and 7.3% had four or more resistance tests. The mean number of tests done per person was lower in Eastern Europe compared to other regions ($P < 0.001$; mean = 0.6 compared to mean = 1.2 in Northern Europe).

The proportion of individuals with a resistance test around the time of virological failure increased from just 2% in 1997 to 29% in 2004 [Unadjusted OR (uOR) 0.08 comparing 1997–1998 to 2003–2004, 95% CI 0.07–0.10, $P < 0.001$] and then declined back to 7.7% in 2012 (uOR 0.22 comparing 2011–2012 to 2003–2004, 95% CI 0.17–0.28, $P < 0.001$), as can be seen in Fig. 1a.

Multivariable models of factors associated with having a resistance test

The association between calendar-year of virological failure and the probability of having a resistance test was confirmed in multivariable analysis (global $P < 0.001$,

Table 1. Baseline characteristics of the study population.

		Total experiencing VF [*N* (%)]	Did not have resistance test [*N* (%)]	Had resistance test [*N* (%)]	*P*[b]
Total		8469 (100)	5793 (100)	2676 (100)	0.006
Sex	Male	6344 (74.9)	4288 (74.0)	2056 (76.8)	0.03
	Female	2125 (25.1)	1505 (26.0)	620 (23.2)	
Ethnicity	White	7305 (86.3)	5030 (86.8)	2275 (85.0)	
	Non-white	1164 (13.7)	763 (13.2)	401 (15.0)	
Risk group	MSM	3401 (40.2)	2212 (38.2)	1189 (44.4)	<0.001
	IDU	2068 (24.4)	1530 (26.4)	538 (20.1)	
	Heterosexual	2381 (28.1)	1640 (28.3)	741 (27.7)	
	Other	619 (7.3)	411 (7.1)	208 (7.8)	
Region[a]	Southern	2639 (31.2)	1856 (32.0)	783 (29.3)	<0.001
	Central	2175 (25.7)	1382 (23.9)	793 (29.6)	
	Northern	2090 (24.7)	1296 (22.4)	794 (29.7)	
	Central Eastern	743 (8.8)	560 (9.7)	183 (6.8)	
	East	607 (7.2)	541 (9.3)	66 (2.5)	
On cART	No	1284 (15.2)	889 (15.3)	395 (14.76)	0.49
	Yes	7185 (84.8)	4904 (84.7)	2281 (85.2)	
		Median (IQR)	Median (IQR)	Median (IQR)	
Age	Year	38 (33.0–44.7)	38 (33.0–44.9)	38 (33.0–44.3)	0.44
$CD4^+$	cells/μl	289 (167.0–446.0)	299 (171.0–459.0)	271 (157.0–420.0)	<0.001
RNA	copies/ml	4233 (1200–24000)	3599 (1080–21200)	6072 (1600–29000)	<0.001

cART, combination antiretroviral therapy; IQR, inter-quartile range; VF, virological failure.
[a]Results from Argentina (*N* = 215) not presented in this study to preserve the anonymity of individual EuroSIDA countries.
[b]*P* values compare those with and without a resistance test, and are calculated using a chi-square test (categorical variables) and a Kruskal–Wallis test (continuous variables).

Supplementary Digital Content 4, http://links.lww.com/QAD/A700), and adjustment for potential confounders had only a limited influence on the calendar-year effect estimates.

Compared to Southern Europe, individuals were more likely to be tested for resistance at or after virological failure in Northern Europe (aOR 2.15, 95% CI 1.96–2.36, $P < 0.001$) and Central Western Europe (aOR 1.66, 95% CI 1.51–1.82, $P < 0.001$). In contrast, individuals in Eastern Europe were less likely to be tested (aOR 0.72, 95% CI 0.55–0.94, $P = 0.02$) compared to individuals in Southern Europe. As expected, individuals with RNA levels of 1000–10 000 copies/ml at virological failure were more likely to have a resistance test compared to individuals with lower viral loads (aOR 2.10, 95% CI 1.86–2.37, $P < .001$ compared to 500–999 copies/ml, Supplementary Digital Content 2, http://links.lww.com/QAD/A700). Due to a small number of individuals being tested for resistance per region and per calendar-year, we did not perform a formal interaction test as this would be underpowered and restricted by empty cells in the regression model. However, plotting the time-trends by region showed that both the rise and decline in resistance testing following virological failure was somewhat more marked in Northern, Central Western and Southern Europe compared to Central Eastern and Eastern Europe (Fig. 1b), although the numbers were limited.

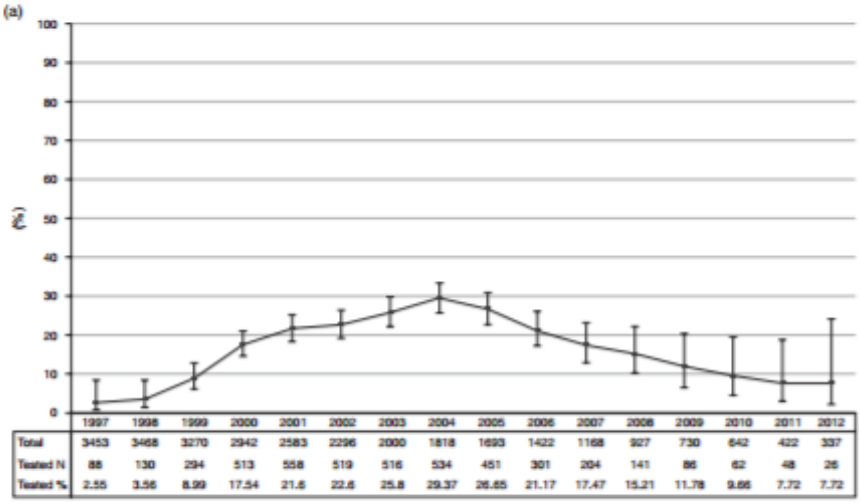
Detected drug resistance

Trends in proportion of individuals with detected drug resistance over time

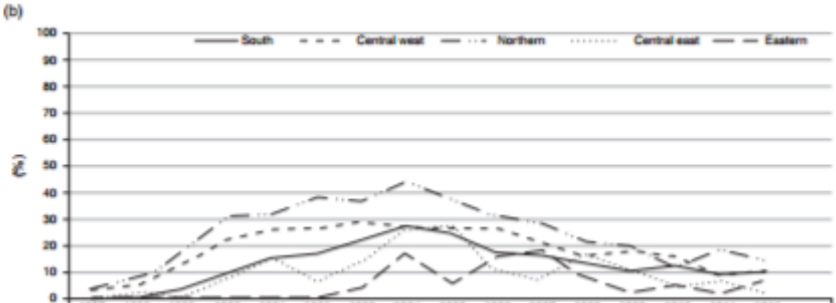
In total, 2431 (77.9%) of the 3119 resistance tests with genotypic data detected drug resistance. The prevalence of mutations with more than 10% prevalence is shown in Fig. 2. Overall, NRTI resistance was most commonly detected in 70.3% of the tests, followed by NNRTI (51.6%) and PI (46.1%) resistance. The most commonly detected individual mutations were M184V (46.3%, NRTI), K103NS (23.4%, NNRTI) and L90M (26.8%, PI). Changes in the proportion of individuals with detected drug resistance each year can be seen in Fig. 3, both overall and after stratification by drug class. Univariable models indicated that calendar-year was associated with the detection of drug resistance (global $P < 0.001$). This trend was not linear, and the prevalence appeared to increase until 2003–2004 followed by somewhat of a decrease. In 1997, just less than two-thirds of the population had detected resistance, and this was somewhat higher (84%) in 2003. In 2012, an estimated 79% of the individuals had detected drug resistance. Looking at univariable specific contrasts, we found strong evidence (all $P < 0.01$) that the odds of detecting resistance were lower in 1997–1998, 1999–2000 and 2009–2010 as compared to 2003–2004 (Table 2).

Multivariable models of factors associated with detected drug resistance

The odds of detecting drug resistance varied by calendar time also after adjustment for confounding (global $P < 0.001$), with the odds of detecting any resistance being lower before and after 2003–2004 (Table 2). Individuals were less likely to have resistance detected in Northern (aOR 0.29, 95% CI 0.21–0.39, $P < .001$) and Central Eastern Europe (aOR 0.47, 95% CI 0.29–0.76, $P = 0.002$) compared to Southern Europe. A number of other factors were independently associated with the risk



	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012
Total	3453	3468	3270	2942	2583	2296	2000	1818	1693	1422	1168	927	730	642	422	337
Tested N	88	130	294	513	558	519	516	534	451	301	204	141	86	62	48	26
Tested %	2.55	3.56	8.99	17.54	21.6	22.6	25.8	29.37	26.65	21.17	17.47	15.21	11.78	9.66	7.72	7.72



	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012
South N	0	7	50	112	159	156	168	206	173	100	57	40	22	18	12	8
South %	0	0.5	3.8	9.3	15.3	16.9	22.2	27.4	24.8	17.4	16.2	13.2	10.3	12.5	9.2	9.8
Central west N	38	54	116	176	185	159	158	123	106	91	59	28	23	16	8	4
Central west %	3.3	5	12.3	22	26.1	26.6	28.9	27	26.8	26.8	22.4	15.7	17.8	15.8	9.3	8.3
Northern N	50	66	127	209	181	188	149	143	108	70	59	34	23	9	12	6
Northern %	4.1	6.5	15.6	30.3	31.8	38.1	36.8	43.9	36.2	31.3	28.6	21.7	19.3	11	18.5	14.6
Central east N	0	3	1	13	29	13	27	51	49	15	10	18	10	5	6	1
Central east %	0	2.5	0.7	7.1	29	6.5	13.7	26.2	27.8	10.1	7	16.7	11.1	4.6	6.7	2.2
Eastern N	0	0	0	0	0	0	12	8	3	12	14	12	4	8	5	7
Eastern %	0	0	0	0	0	0	4.1	17.4	5.7	15.6	17.5	9	2.8	4.7	2.4	6.4

Fig. 1. Proportion of individuals with a resistance test following VF over time (a) and per region (b).

of resistance detection. Individuals with a history of mono/dual therapy were more likely to have detected drug resistance (aOR 1.54 vs. those who started cART from ART naive, 95% CI 1.14–2.08, $P=0.007$), as were individuals who had experienced virological failure previously (aOR 1.85 vs. those who experienced virological failure for the first time, 95% CI 1.40–2.45, $P<0.001$). Individuals with RNA levels between 1000 and 10 000 copies/ml were more likely to have detected resistance (aOR 1.63, compared to individuals with RNA levels less than 1000 95% CI 1.19–2.23, $P=0.002$), but individuals with very high RNA levels (>50 000 copies/ml) were not significantly more likely to have detected resistance (aOR 1.20, 95% CI 0.84–1.72)

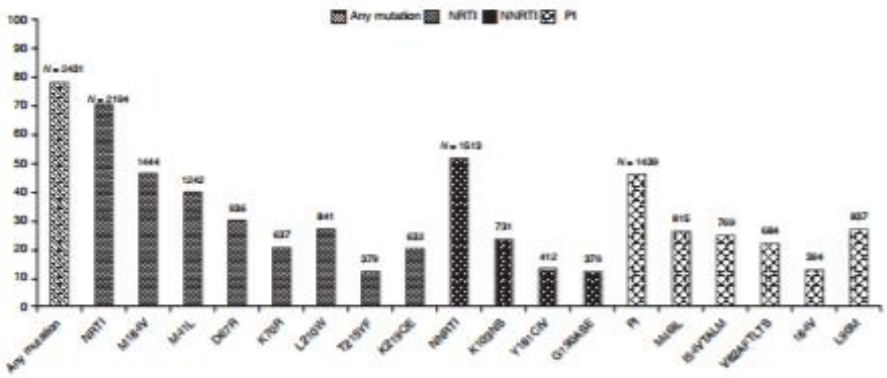


Fig. 2. Prevalence of resistance mutations with > 10% prevalence, per resistance test.

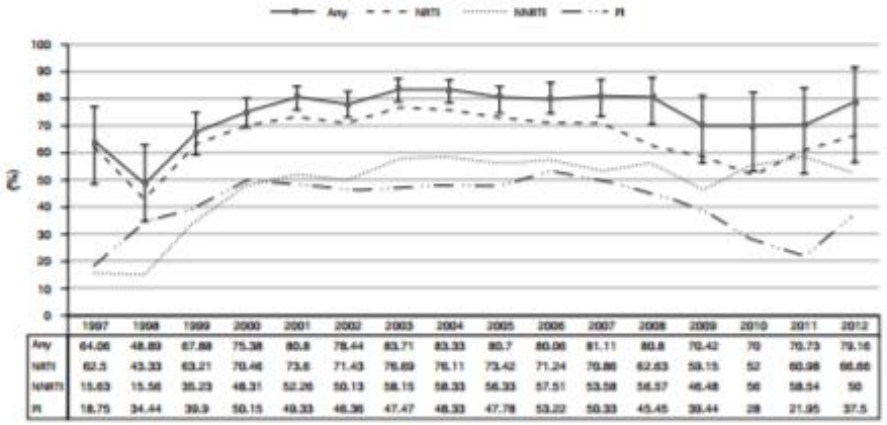
compared to individuals with RNA levels less than 1000. Findings were broadly consistent when conducting the analysis for each drug class separately (data not shown).

Sensitivity analyses

The estimates of the prevalence of virological failure and resistance in a number of sensitivity analyses are shown in Supplementary Digital Content 5 (http://links.lww.com/QAD/A700). Briefly, the proportion of individuals with a resistance test was higher when using stricter criteria to define virological failure, including only considering virological failures that were followed by a switch in regimen, but remained below 50%. The proportion of resistance tests with detected drug resistance remained reasonably stable despite using stricter definitions of virological failure. Multivariable results from these sensitivity analyses were in broad agreement with the results described above (data not shown).

Discussion

In this analysis of a large cohort of HIV-positive individuals with virological failure from across Europe, around one-third of the individuals received a resistance test within 12 months of virological failure. This proportion decreased after 2004. The relatively low proportion of individuals in our study receiving a resistance test at or after virological failure extend and confirm previous EuroSIDA findings [21], and indicate that there is a potential discrepancy between clinical



	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012
Any	64.06	48.89	67.89	75.38	80.8	78.44	83.71	83.33	80.7	80.06	81.11	80.8	70.42	70	70.73	79.16
NRTI	62.5	43.33	63.21	70.46	73.6	71.43	76.69	76.11	73.42	71.24	70.86	62.63	59.15	52	60.98	66.66
NNRTI	15.63	15.56	35.23	48.31	52.26	50.13	58.15	58.33	56.33	57.51	53.58	56.57	46.48	56	58.54	50
PI	18.75	34.44	39.9	50.15	49.33	46.36	47.47	48.33	47.78	53.22	50.33	45.45	39.44	28	21.95	37.5

Fig. 3. Prevalence of detected resistance over time, according to drug class.

Table 2. Factors associated with having detected resistance (any class).

		Resistance		Unadjusted		Adjusted[a,b]		Global P value
		N	%	OR (95% CI)	P	OR (95% CI)	P	
Age (years)	<35	325	72.54	1.00		1.00		
	35–40	584	78.1	1.35 (1.01–1.80)	0.04	1.13 (0.82–1.55)	0.45	0.95
	45–50	571	79.5	1.47 (1.09–1.99)	0.01	1.06 (0.75–1.50)	0.73	
	50–55	667	78.8	1.41 (1.05–1.90)	0.02	1.11 (0.78–1.57)	0.57	
	>55	284	79.1	1.43 (0.99–2.07)	0.05	1.13 (0.75–1.72)	0.55	
Sex	Male	1884	79.2	1.00		1.00		
	Female	547	74.0	0.75 (0.60–0.94)	0.01	0.74 (0.55–0.98)	0.04	0.04
Ethnic group	White	1981	78.4	1.00		1.00		
	Non-white	450	75.9	0.87 (0.67–1.12)	0.28	0.94 (0.70–1.26)	0.66	0.67
Risk group	MSM	1068	78.1	1.00		1.00		
	IDU	463	76.2	0.90 (0.69–1.17)	0.43	0.69 (0.51–0.95)	0.02	0.04
	Heterosexual	710	77.7	1.04 (0.82–1.32)	0.75	1.12 (0.82–1.53)	0.48	
	Other	195	79.0	1.05 (0.71–1.56)	0.79	0.93 (0.60–1.44)	0.76	
Region	South	700	87.2	1.00				
	Central Western	844	83.5	0.74 (0.55–1.00)	0.05	0.81 (0.58–1.13)	0.22	<0.0001
	North	694	65.4	0.28 (0.21–0.37)	<0.001	0.29 (0.21–0.39)	<.001	
	Central Eastern	106	74.7	0.43 (0.27–0.69)	<0.001	0.47 (0.29–0.76)	0.002	
	East	43	81.1	0.63 (0.31–1.29)	0.21	1.03 (0.46–2.32)	0.94	
History of mono/dual therapy	No	353	70.3	1.00				
	Yes	2078	79.4	1.63 (1.31–2.01)	<0.001	1.54 (1.14–2.08)	0.004	0.007
Subtype	B	1555	78.3	1.00				
	Non-B	328	73.9	0.78 (0.59–1.04)	0.09	0.80 (0.57–1.10)	0.17	0.23
	Unknown	548	79.4	1.07 (0.84–1.36)	0.60	0.83 (0.63–1.09)	0.18	
RNA at failure	<1000	205	69.3	1.00				
	1000–10000	780	79.0	1.67 (1.25–2.24)	<0.001	1.63 (1.19–2.23)	0.002	0.002
	10000–50000	941	80.8	1.86 (1.39–2.51)	<0.001	1.68 (1.22–2.31)	0.002	
	>50000	505	74.3	1.35 (0.98–1.85)	0.06	1.20 (0.84–1.72)	0.32	
CD4+ at failure, per 100 increase[b]		n/a		0.98 (0.95–1.02)	0.39	0.95 (0.91–0.99)	0.01	0.02
Previous resistance test	No	1455	77.5	1.00				
	Yes	976	78.7	1.07 (0.89–1.28)	0.46	0.88 (0.71–1.09)	0.23	0.23
Previous virological failure	No	249	61.8	1.00				
	Yes	2182	80.3	2.53 (2.02–3.15)	<0.001	1.85 (1.40–2.45)	<.001	<0.0001
Calendar-year	1997–1998	85	55.2	0.24 (0.16–0.36)	<0.001	0.31 (0.19–0.49)	<.001	<0.0001
	1999–2000	376	72.6	0.52 (0.39–0.69)	<0.001	0.47 (0.34–0.65)	<.001	
	2001–2002	594	79.6	0.77 (0.60–1.00)	0.05	0.75 (0.57–0.99)	0.04	
	2003–2004	598	83.5	1.00				
	2005–2006	442	80.5	0.82 (0.62–1.08)	0.15	0.73 (0.55–0.98)	0.03	
	2007–2008	203	81.2	0.85 (0.59–1.23)	0.39	0.90 (0.60–1.35)	0.61	
	2009–2010	85	70.3	0.47 (0.30–0.72)	<0.001	0.49 (0.30–0.79)	0.003	
	2011–2012	48	73.9	0.56 (0.31–0.99)	0.05	0.68 (0.36–1.27)	0.23	

CI, confidence interval; OR, odds ratio.
[a]The multivariable models were adjusted for all the factors listed in the table.
[b]Three individuals were excluded from the model with CD4+ cell count as an exposure as well as the multivariable model due to missing CD4+ cell counts.

practice and current guidelines which recommend to always test for resistance after virological failure.

Several clinical trials have shown a direct clinical benefit of resistance testing [10–13], and it is important to encourage the use of genotyping by clinicians. However, clinical decisions are complicated by numerous factors and it is not uncommon that guidelines are not followed exactly in real life. The fact that the proportion tested for resistance is higher when considering virological failure followed by a switch in a sensitivity analysis is reassuring, as it indicates that clinicians may test selectively those individuals they are considering switching to a different drug class. Adjustment for a range of clinical variables did not affect the declining trend observed after 2004; however, we cannot rule out that this result might have been different if we could control for other unmeasured factors that could influence clinicians' decisions to order a resistance, such as an increased availability of different drugs. In addition, the marked reduction in the proportion of individuals experiencing virological failure over time documented here indicates that virological failure is becoming less common. It could be that when seen in a clinical setting, the reason for the virological failure may be put down to poor adherence, a chaotic lifestyle or personal issues rather than drug resistance. This is in agreement with the higher proportion of adherent individuals having a resistance test observed in the sensitivity analyses. However, it should be noted that the adherence data available is of limited scope, and findings should therefore be interpreted with caution.

Other studies of trends in resistance testing have found conflicting results. A recent study of drug resistance

prevalence in Sweden provided data on the number of tests done over time, and showed that these remained relatively stable [23]. This also appears to be the case in the United States, at least from 2003 onwards [24]. Nonetheless, our results are in broad agreement with a study conducted in British Columbia, Canada, from 2011, which found both under-utilization of resistance testing as well as a lower probability of testing after 2004 [25]. An analysis of data from the UK Collaborative HIV Cohort Study cohort has shown that 46% of individuals had a resistance test after viral rebound prior to a change in therapy, similar to the estimate of 47% we found when defining failure according to whether or not a high viral load was followed by a switch in the regimen [26].

Despite a declining trend in resistance testing, a relatively high proportion – almost 80% – of tests detected any drug resistance. This estimate is comparable to those obtained from other European cohorts [27], but somewhat higher than data obtained from the United Kingdom [28], potentially due to differences in the populations studied. The fact that a relatively high proportion of resistance tests did detect drug resistance could indicate that clinicians may be taking a selective approach to resistance testing, whereby those individuals judged most likely to have resistance are also the ones offered a test.

The proportion of tests detecting any resistance peaked in 2003–2004, and there was some evidence to suggest that this proportion had declined in the more recent calendar-years. A decline in the prevalence of resistance in recent years among individuals experiencing virological failure has been found in a number of European cohorts [22,23,28,29] and also in the USA [30]. Such a decrease could be explained by improvements in the potency of drugs used, minimized side effects and an increase in therapeutic options that have all lowered the risk of developing drug resistance [22,29]. Simplified drug regimens that are easier to take, combined with efforts to educate patients, and the development and use of drugs with a high genetic barrier may also play an important role. Taken together with the reduced proportion of individuals experiencing virological failure in more recent calendar-years, the current analysis documents a marked reduction in the number of individuals experiencing virological failure with acquired drug resistance.

Regional differences in the probability of both receiving a resistance test and detecting drug resistance were observed. Clinicians in Northern Europe were the most likely to test for resistance following virological failure, but also least likely to detect any drug resistance once the test was prescribed. In contrast, clinicians in Eastern Europe were comparatively less likely to test for resistance following virological failure compared to Central Western and Northern Europe, but also more likely to detect resistance when doing a test compared to Central Eastern and Northern Europe. As it is unlikely that the biological probability of developing drug resistance in a situation of virological failure differs according to region, it is possible that the lower testing rates in Eastern Europe are causing individuals to be maintained on failing therapies for longer, thus leading to the development of resistance. Clinicians in Eastern Europe may also be more selective about who they test for resistance, as indicated in a recent EuroSIDA survey [31].

Our findings should be interpreted with caution as the analysis was subject to several limitations. First of all, the definition of virological failure referred to by guidelines for resistance testing has changed over time. We attempted to address this possible bias in sensitivity analyses, and although the proportion receiving a test increased with more stringent definitions of virological failure, it still remained relatively low. Furthermore, we cannot rule out under-reporting of the numbers of tests ordered and performed from the clinical sites, although rigorous efforts are made to minimize such under-reporting by quality control visits. Resistance tests done in more recent years might also be subject to reporting delay, which similarly may have led to an underestimation of the number of tests done after a certain calendar period. We furthermore cannot exclude that some instances of virological failure have been misclassified. It is possible that some of the virological failures, particularly early on during follow-up, may be due to treatment interruptions. Finally, EuroSIDA clinics may not be representative of all HIV clinics in Europe, as some countries are represented by relatively few centres. It should also be noted that the prevalence of detected drug resistance in this study differs from the true population level prevalence of drug resistance, which requires genotypic data from all individuals on ART or some estimation of the prevalence of resistance in those who were not tested. We have taken this latter approach in one of our previous publications, which provided an estimate of the overall population level prevalence of resistance in 1 year (i.e. 2008) [32].

To conclude, our findings indicate that the clinical approach to resistance testing may diverge from that laid out in guidelines, and we observed calendar-year and regional differences both in resistance testing and the probability of detecting resistance. Public health policy aimed at minimizing the emergence of drug resistance might benefit from targeting specific regions of Europe, and efforts to minimize inter-regional differences in the availability and utilization of resistance testing in the European region may be warranted.

Acknowledgements

Author contributions: A.C.L., J.D.L. and A.S. designed the study and analysis plan. A.S. performed the statistical

Appendix VII: Conference Presentation arising from Chapter 4

15th European AIDS Conference 2015

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Long-term Virological Outcomes of ART-experienced Patients Receiving Raltegravir in a Large European Cohort Study

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BACKGROUND

The use of integrase inhibitors such as Raltegravir (RAL) has recently increased in both ART-experienced and ART-naive individuals. Although data from clinical trials suggest good long-term response rates, evaluations of long-term outcomes using routinely collected data is important as it can provide a 'real-life' picture, often with greater generalisability.

AIM:

- Describe long-term virological outcomes of individuals receiving RAL

METHODS

We used data from the EuroSIDA cohort: a large cohort of almost 19,000 individuals from across Europe, Israel and Argentina. The study regularly collects both CD4 counts and viral loads, details on ART use including start and stop dates and resistance data. Individuals were included if they started RAL as ART-experienced for the first time after 1/1/2006, and had at least one month of follow-up and a baseline HIV-RNA measurement available. Virological failure was defined in different ways depending on the baseline level of viral suppression:

- ***If baseline VL ≤ 50:*** Confirmed VL≥200
- ***If baseline VL>50:*** Confirmed VL≥200 after 6 months of receiving RAL
- ***Irrespective of baseline VL:*** Discontinuation of RAL with the main reason for stopping given by the treating physician as being VF

Time to and risk factors for VF identified using Kaplan-Meier plots and Cox Regression models, and all analyses were conducted both as:

- ***On Treatment:*** Individuals' follow-up lasted until they failed RAL, stopped RAL or their last clinic visit, whichever came first.
- ***Intention to Treat:*** Individuals follow-up lasted until they failed RAL or their last available clinic visit, whichever came first.

All analyses were stratified according to baseline VL (>50 or ≤50), as we hypothesised that the probability of virological failure would be different among individuals who were switched in order to control viral replication, and individuals who were switched in order to simplify the regimen or to avoid certain side-effects.

RESULTS

1796 individuals could be included: the majority started RAL with a supressed viral load ***(Table 1)***. A large variety of drugs, over 693 combinations, were used with Raltegravir. The majority of people received at least 3 drugs, and the most common combinations can be seen in ***Table 1***. Baseline resistance profiles can be seen in ***Figure 1***; data on integrase resistance was not available.

In on treatment analysis 126 individuals experienced VF during their FU, which equals a cumulative probability of VF at 20% by 6 years (baseline VL>50) and 7% by 6 years (baseline VL ≤50; ***Figure 2a***). This was similar, but slightly higher in intention to treat analyses (***Figure 2b***): 24% by 6 years (baseline VL>50) and 11% by 6 years (baseline VL ≤50. Risk factors for VF can be seen in ***Figure 3***.

LIMITATIONS

The study has some limitations. Firstly, the fact that we do not collect a fully validated measure of adherence in EuroSIDA means that we cannot capture or assess the influence of poor adherence on our results. Secondly, these failure probabilities may be difficult to put into context, as there is no clear control group that we can use as a comparison arm. And finally, we were only able to describe risk factors for VF among individuals starting RAL with a raised VL, as the number of events in those starting with a supressed viral load was low.

CONCLUSIONS:

- Up to 11% of ART experienced individuals starting Raltegravir with a supressed VL experienced VF by 6 years.
- Among those who started with a raised VL, up to 24% experienced virological failure by 6 years
- In this latter group, the determinants of virological failure we could identify were baseline CD4, geographical region and calendar year.

FUTURE WORK

We are currently genotyping individuals who experienced VF to describe integrase resistance patterns following VF.

Download poster at: www.chip.dk

Appendix VIII: Conference Presentation arising from Chapter 5

Poster No. P205 HIV Glasgow Drug Therapy 2014

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Detection of Resistance Mutations and CD4 Slopes in Individuals Experiencing Sustained Virological Failure

A Schultze[1], R Paredes[2], C Sabin[1], AN Phillips[1], D Pillay[3], O Kirk[5], A Pozniak[4], M Nelson[4], J Lundgren[5] and A Cozzi-Lepri[1] on behalf of EuroSIDA in EuroCOORD, UK CHIC and UK HDRD

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INTRODUCTION

Several HIV- drug resistance mutations have been shown to affect viral fitness in vitro, and their presence may result in clinical benefit for patients kept on a virologically failing regimen due to an exhaustion of drug options.

AIM

To quantify the effect of resistance mutations on CD4 slopes in patients undergoing episodes of viral failure.

PATIENTS AND METHODS

- The data used come from EuroSIDA and the UK Collaborative HIV Cohort (UK CHIC) study. EuroSIDA contains data on >18,000 individuals from 34 European countries, Israel and Argentina; UK CHIC contains data on >45,000 individuals attending HIV clinics in the UK, whose records are linked to the UK HIV Drug Resistance Database.
- Patients were included in this analysis if they had at least one episode of sustained virological failure (≥3 consecutive RNA measurements >500 copies/ml on ART) with at least 3 CD4 measurements and a resistance test during the episode.
- Mutations were identified using the IAS-USA[1] list, and were presumed to be present from detection until the end of an episode.
- Linear mixed models with a random intercept and slope were used to estimate CD4 slopes.
- Individual mutations with a population prevalence of >10% were tested for their association with CD4 slope.

RESULTS

- 2731 patients experiencing a median of 1 (range 1-4) episodes were included. Baseline characteristics can be seen in **Table 1**.
- Overall, CD4 counts declined by 17.1 (-19.7; -14.5) cells per year; this decline was less marked when viral suppression was higher (current HIV RNA more than 1.5 log below the setpoint; p=0.01).
- In multivariable models adjusting for viral load, CD4 decline was slower during episodes with detected resistance (21.0 cells/year less, 95%CI=11.7-30.3) compared to episodes without detected resistance, p<0.001; **Figure 1**).
- Among those with more than 1 resistance mutation, there was only weak evidence that class-specific mutations had any effect on the CD4 slope (**Figure 1**).
- The effects of individual mutations were explored, but none were significantly associated with the CD4 slope (**Table 2-3**).

CONCLUSIONS

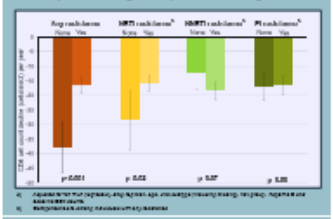
In our study population, presence of resistance mutations was associated with less steep CD4 declines. This may be due to a biological effect of resistance on CD4 slopes, or other unmeasured factors such as poor adherence among individuals without resistance. Among individuals with detected drug resistance, we found no evidence suggesting that the presence of individual mutations was associated with beneficial CD4 slope changes.

1. Victoria A. Johnson, MD; Vincent Calvez, MD, PhD; Huldrych F. Günthard, MD; Roger Paredes, MD, PhD; Deenan Pillay, MD, PhD; Robert W. Shafer, MD; Annemarie Wensing, MD, PhD; Douglas D. Richman, MD. Update of the Drug Resistance Mutations in HIV-1: March 2013. *Top. Antivir. Med.* **21**, 6–14 (2013)

Baseline Characteristics

		N	%
Total		2731	>00
Gender	Male	2179	79.8
Ethnicity	White	2003	73.3
Infection Mode	MSM	1541	56.4
	IDU	272	1.0
	Heterosexual	754	27.6
Age	Median, IQR	39	33, 45
CD4 (cells/mm3)	Median, IQR	200	140, 264
HIV RNA (cp/ml)	Median, IQR	14100	3980, 69100
For each episode:		N	%
Duration of episode	Median, IQR	15	9, 27
OBS	Median, IQR	1	0, 2
Any resistance		2779	68.4
NRTI		2455	78.5
NNRTI		1862	62.1
PI		1920	48.4

CD4 slope* according to the presence of drug resistance



Association between NRTI mutations and CD4 counts*

	Unadjusted		Adjusted*	
	Difference in Slope (95% CI)	p	Difference in Slope (95% CI)	p
N118V	1.28 (-4.89, 7.21)	0.67	4.12 (-1.85, 10.09)	0.18
M50R	-3.08 (-19.89, 12.99)	0.68	-4.65 (-21.66, 11.84)	0.57
L74V	-4.89 (-11.84, 2.26)	0.18	-3.54 (-10.84, 3.88)	0.33
M41L	-1.03 (-8.84, 4.48)	0.71	-1.88 (-7.70, 3.95)	0.58
D67N	-1.23 (-8.64, 4.18)	0.66	0.22 (-5.21, 5.64)	0.94
K70R	-1.53 (-7.39, 4.32)	0.61	3.44 (-2.43, 9.31)	0.25
L210W	0.29 (-5.17, 5.75)	0.92	-2.08 (-7.95, 3.41)	0.46
T215Y	0.39 (-6.06, 6.83)	0.89	-0.22 (-6.88, 6.24)	0.94
K219Q	2.32 (-4.62, 9.28)	0.51	4.48 (-2.47, 11.44)	0.21
V118I	0.42 (-5.32, 6.17)	0.89	-1.06 (-7.73, 3.61)	0.51

Association between NNRTI/PI mutations and CD4 counts*

	Unadjusted		Adjusted*	
	Difference in Slope (95% CI)	p	Difference in Slope (95% CI)	p
NNRTI mutations				
K103N	-0.79 (-6.50, 4.94)	0.79	-1.61 (-7.15, 4.33)	0.64
Y181C	0.85 (-5.53, 7.22)	0.79	1.89 (-5.26, 7.44)	0.75
G190A	-0.79 (-7.88, 6.10)	0.82	-1.32 (-8.22, 5.58)	0.71
PI mutations				
M46I	-0.93 (-6.81, 5.15)	0.79	-1.07 (-7.06, 4.93)	0.73
V82A	2.06 (-3.87, 7.97)	0.50	2.26 (-3.68, 8.21)	0.49
L90M	-2.74 (-8.23, 2.75)	0.33	-3.17 (-8.28, 1.74)	0.18

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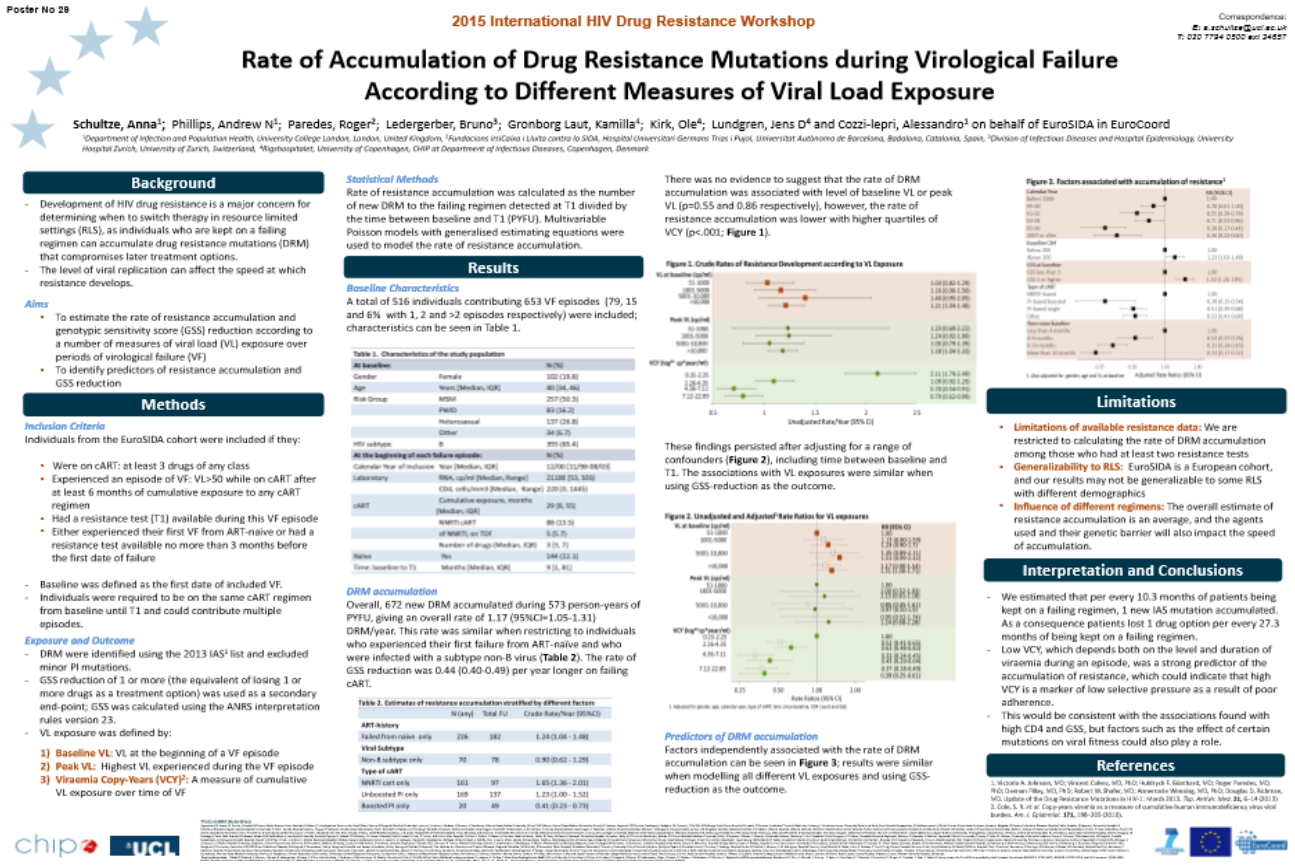
Appendix IX: Conference Presentation arising from Chapter 6



Poster No 29

2015 International HIV Drug Resistance Workshop

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Rate of Accumulation of Drug Resistance Mutations during Virological Failure According to Different Measures of Viral Load Exposure

Schultze, Anna[1]; Phillips, Andrew N[1]; Paredes, Roger[2]; Ledergerber, Bruno[3]; Gronborg Laut, Kamilla[4]; Kirk, Ole[4]; Lundgren, Jens D[4] and Cozzi-Iepri, Alessandro[1] on behalf of EuroSIDA in EuroCoord

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Background

- Development of HIV drug resistance is a major concern for determining when to switch therapy in resource limited settings (RLS), as individuals who are kept on a failing regimen can accumulate drug resistance mutations (DRM) that compromises later treatment options.
- The level of viral replication can affect the speed at which resistance develops.

Aims

- To estimate the rate of resistance accumulation and genotypic sensitivity score (GSS) reduction according to a number of measures of viral load (VL) exposure over periods of virological failure (VF)
- To identify predictors of resistance accumulation and GSS reduction

Methods

Inclusion Criteria

Individuals from the EuroSIDA cohort were included if they:

- Were on cART: at least 3 drugs of any class
- Experienced an episode of VF: VL>50 while on cART after at least 6 months of cumulative exposure to any cART regimen
- Had a resistance test (T1) available during this VF episode
- Either experienced their first VF from ART-naive or had a resistance test available no more than 3 months before the first date of failure

- Baseline was defined as the first date of included VF.
- Individuals were required to be on the same cART regimen from baseline until T1 and could contribute multiple episodes.

Exposure and Outcome

- DRM were identified using the 2013 IAS[2] list and excluded minor PI mutations.
- GSS reduction of 1 or more (the equivalent of losing 1 or more drugs as a treatment option) was used as a secondary end-point; GSS was calculated using the ANRS interpretation rules version 23.
- VL exposure was defined by:

1) **Baseline VL**: VL at the beginning of a VF episode
2) **Peak VL**: Highest VL experienced during the VF episode
3) **Viraemia Copy-Years (VCY)**[2]: A measure of cumulative VL exposure over time of VF

Statistical Methods

Rate of resistance accumulation was calculated as the number of new DRM to the failing regimen detected at T1 divided by the time between baseline and T1 (PYFU). Multivariable Poisson models with generalised estimating equations were used to model the rate of resistance accumulation.

Results

Baseline Characteristics

A total of 516 individuals contributing 653 VF episodes (79, 15 and 6% with 1, 2 and >2 episodes respectively) were included; characteristics can be seen in Table 1.

Table 1. Characteristics of the study population

At baseline:		N (%)
Gender	Female	102 (19.8)
Age	Years (Median, IQR)	40 (34, 46)
Risk Group	MSM	257 (50.3)
	PWID	83 (16.2)
	Heterosexual	137 (26.8)
	Other	34 (6.7)
HIV subtype	B	355 (85.4)
At the beginning of each failure episode:		N (%)
Calendar Year of Inclusion	Year (Median, IQR)	11/00 (11/99-08/03)
Laboratory	RNA, cp/ml (Median, Range)	21100 (53, 500)
	CD4, cells/mm3 (Median, Range)	220 (0, 1445)
cART	Cumulative exposure, months (Median, IQR)	29 (8, 55)
	NNRTI-cART	88 (13.5)
	of NNRTI, on TDF	5 (5.7)
	Number of drugs (Median, IQR)	3 (3, 7)
Naïve	Yes	144 (22.1)
Time: baseline to T1	Months (Median, IQR)	9 (3, 81)

DRM accumulation

Overall, 672 new DRM accumulated during 573 person-years of PYFU, giving an overall rate of 1.17 (95%CI=1.05-1.31) DRM/year. This rate was similar when restricting to individuals who experienced their first failure from ART-naïve and who were infected with a subtype non-B virus (**Table 2**). The rate of GSS reduction was 0.44 (0.40-0.49) per year longer on failing cART.

Table 2. Estimates of resistance accumulation stratified by different factors

	N (any)	Total FU	Crude Rate/Year (95%CI)
ART-history			
Failed from naive only	226	182	1.24 (1.04 - 1.48)
Viral Subtype			
Non-B subtype only	70	78	0.90 (0.62 - 1.29)
Type of cART			
NNRTI cart only	161	97	1.65 (1.36 - 2.01)
Unboosted PI only	168	137	1.23 (1.00 - 1.52)
Boosted PI only	20	49	0.41 (0.25 - 0.71)

There was no evidence to suggest that the rate of DRM accumulation was associated with level of baseline VL or peak VL (p=0.55 and 0.86 respectively), however, the rate of resistance accumulation was lower with higher quartiles of VCY (p<.001; **Figure 1**).

Figure 1. Crude Rates of Resistance Development according to VL Exposure

These findings persisted after adjusting for a range of confounders (**Figure 2**), including time between baseline and T1. The associations with VL exposures were similar when using GSS-reduction as the outcome.

Figure 2. Unadjusted and Adjusted[1] Rate Ratios for VL exposures

Predictors of DRM accumulation

Factors independently associated with the rate of DRM accumulation can be seen in **Figure 3**; results were similar when modelling all different VL exposures and using GSS-reduction as the outcome.

Figure 3. Factors associated with accumulation of resistance[1]

Limitations

- **Limitations of available resistance data:** We are restricted to calculating the rate of DRM accumulation among those who had at least two resistance tests
- **Generalizability to RLS:** EuroSIDA is a European cohort, and our results may not be generalizable to some RLS with different demographics
- **Influence of different regimens:** The overall estimate of resistance accumulation is an average, and the agents used and their genetic barrier will also impact the speed of accumulation.

Interpretation and Conclusions

- We estimated that per every 10.3 months of patients being kept on a failing regimen, 1 new IAS mutation accumulated. As a consequence patients lost 1 drug option per every 27.3 months of being kept on a failing regimen.
- Low VCY, which depends both on the level and duration of viraemia during an episode, was a strong predictor of the accumulation of resistance, which could indicate that high VCY is a marker of low selective pressure as a result of poor adherence.
- This would be consistent with the associations found with high CD4 and GSS, but factors such as the effect of certain mutations on viral fitness could also play a role.

References

1. Victoria A. Johnson, MD; Vincent Calvez, MD, PhD; Huldrych F. Günthard, MD; Roger Paredes, MD, PhD; Deenan Pillay, MD, PhD; Robert W. Shafer, MD; Annemarie Wensing, MD, PhD; Douglas D. Richman, MD. Update of the Drug Resistance Mutations in HIV-1: March 2013. Top. Antivir. Med. **21**, 6–14 (2013)
2. Cole, S. R. et al. Copy-years viremia as a measure of cumulative human immunodeficiency virus viral burden. Am. J. Epidemiol. **171**, 198–205 (2010).

Appendix X: Conference Presentation arising from Chapter

Poster No 16-966

International HIV Drug Resistance Workshop and CROI 2016

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CROI abstract number: 486

Influence of Transmitted Drug Resistance on CD4 Decline among ART Naïve HIV Patients

Anna Schultze[1], Carlo Torti[2], Alessandro Cozzi-Lepri[1], Anne-Mieke Vandamme[3,4], Maurizio Zazzi[5], Helen Sambatakou[6], Andrea De Luca[7], Anna Maria Geretti[8], Anders Sonnerborg[9] and Giuseppe Lapadula[10]

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Introduction

- Transmitted drug resistance mutations (TDRM) may lead to an altered progression of HIV disease before the start of antiretroviral therapy (ART).
- Existing research into the effect of the effect of TDRM on the natural history of HIV have found conflicting results.

Aim:

- To investigate the effect of TDRM on CD4 count changes before the start of ART.

Methods

Data and Study population

Data from several European HIV clinics (ViroLAB, EuResist and EuroSIDA contributing clinics; Royal Free and St Mary's Hospital, London; University of Bari) were merged. Individuals were included if they:

- Were older than 18 years old.
- Had at least 1 CD4 count available.
- Had at least 1 genotypic resistance test before starting ART (first date any ART drug was initiated).
- Had data available for the viral set point to be estimated.

FU lasted until the last CD4 measurement before ART. Baseline was defined as the date of the first available CD4 count. TDRM were identified using the WHO 2009 surveillance list[1]. We presumed that mutations detected at any point during follow-up had been present since baseline, and for those with more than one pre-ART resistance test available resistance was considered in a cumulative manner. The set-point was defined as the median of all pre-ART viral load measurements.

Statistical methods

Linear mixed models with a random intercept and slope were used to estimate the effect of TDRM on CD4 slopes. The 10 most commonly detected mutations were tested for their effect on CD4 slopes; for these comparisons we used a Bonferroni corrected p-value threshold of 0.005 to indicate statistical significance.

Sensitivity analyses:

A. The analyses were repeated stratified by subtype B and non-B.
B. The analyses were repeated using the minimum available date for each person as the baseline date, restricting the analyses to those who had this information available (N=1285).

Results

Baseline characteristics

6180 individuals contributing a median of 5 (IQR= 2-9) CD4 measurements over a median of 1 (IQR=0.2-2.7 years) years were included. The baseline characteristics according to the prevalence of TDRM can be seen in **Table 1**. The majority of the individuals were infected with a Subtype B virus (64%).

The median baseline CD4 count was 420 cells/mm3, and the median viral set point was 4.5 $\log_{10}$ cp/ml. Among individuals with TDRM, the median CD4 count was 433 cells/mm^3, and among those with wild-type viruses it was 420 cells/m^3. The viral set point was 4.4 $\log_{10}$ cp/ml among those with TDRM, and 4.5 $\log_{10}$ cp/ml among those without TDRM (**Table 1**). We found no marked changes in CD4 and VL set point according to the class of resistance present (data not shown).

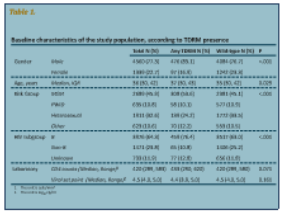
Table 1.
Baseline characteristics of the study population, according to TDRM presence

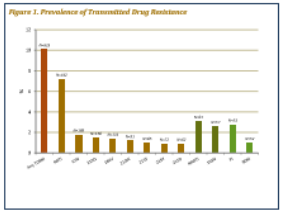
Figure 1. Prevalence of Transmitted Drug Resistance

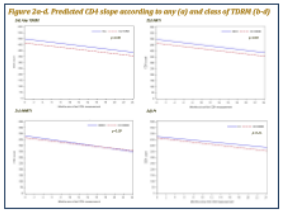
Figure 2a-d. Predicted CD4 slope according to any (a) and class of TDRM (b-d)

Resistance Prevalence

Resistance was detected in 9.8% of individuals; 7.2% had NRTI resistance, 3.1% NNRTI resistance and 2.8% PI resistance. The 10 most commonly detected mutations and their prevalence can be seen in **Figure 1.**

The effect of TDRM on CD4 count changes before the start of ART

The overall estimated CD4 decline was -54.1 cells/mm^3/year (95%CI = -56.3, -51.7). In univariable analyses, we found no evidence that CD4 decline differed according to the presence of any TDRM compared to wild-type (**Figure 2a-d).** There was also no evidence that CD4 count decline differed among individuals with NRTI or PI resistance as compared to those with wild-type viruses. There was some weak evidence suggesting that CD4 counts declined more steeply among individuals with detected NNRTI resistance (difference compared to wild-type= -12 (95%CI=-25,+2) cells/mm^3/year; p=0.08). These conclusions did not change upon adjustment for covariates including viral load set point (**Table 2**).

Associations between individual TDRM and CD4 count changes before the start of ART

The associations between individual TDRM and CD4 slopes can be seen in **Table 3**. There was some suggestion that CD4 slopes were less marked among individuals who had the T215Y mutation (difference compared to wild-type= +35 (95%CI=+15, +56) cells/mm^3/year) but more marked among individuals who had the revertant T215D mutation (difference compared to wild-type= -39 (95%CI= -63, -15) cells/mm^3/year); however, this was not the case for T215S. There was no evidence of an association between the M184V and CD4 count declines (difference compared to wild-type= -+0.35 (-19.48, +20.18) cells/mm^3/year).

Sensitivity analyses

The results from sensitivity analyses can be seen in **Table 4a-b.** When restricting the analysis to individuals with subtype B viruses only, there was still no evidence to suggest that the presence of any TDRM was associated with differences in CD4 decline (p=0.76). Using the minimum date available as the baseline date did not change the overall conclusions.

Limitations

-**Date of Seroconversion:** Due to data availability, we could not use the date of seroconversion as the baseline date. CD4 count trends may differ according to time since seroconversion.
-**Generalizability:** Individuals with a resistance test before starting treatment may differ from individuals not tested. In addition, the natural history of HIV can vary according to the subtype of the virus. These factors could limit the generalisability of our results.
-**Power:** The prevalence of TDRM, and in particular the prevalence of individual mutations, is relatively low. Despite the large dataset our analysis may have suffered from a lack of power.

Conclusions and Future Work

- We were not able to find convincing evidence supporting the hypothesis that the rate of CD4 decline in the absence of ART is different between patients with and without TDRM.
- This could reflect the fact that mutations with less impact on fitness are preferentially transmitted.
- We cannot rule out the fact that TDRM may influence the rate of CD4 decline differently in different time-periods since seroconversion.
- Future work will focus on characterising viral load changes over time, describing associations between TDRM and viral load changes and evaluating our assumption that mutations persisted throughout FU in sensitivity analyses.

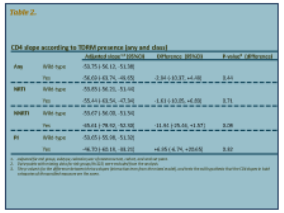
Table 2.
CD4 slope according to TDRM presence (any and class)

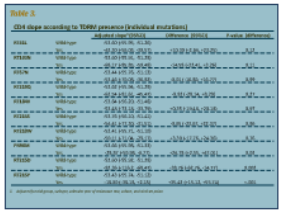
Table 3.
CD4 slope according to TDRM presence (individual mutations)

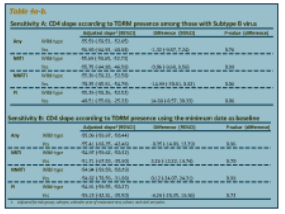
Table 4a-b.
Sensitivity A: CD4 slope according to TDRM presence among those with Subtype B virus
Sensitivity B: CD4 slope according to TDRM presence using the minimum date as baseline

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